

UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	DePhillipo and Ricciardi
Serial No.	09/924,011
Filing Date	August 7, 2001
Art Unit	1633
Confirmation No.	1968
Examiner	Kaushal
Title	Use Of Genetic Information For Detecting a Bone Density Condition
Attorney Docket No.	073981.2

Cincinnati OH

November 16, 2007

**DECLARATION OF ROBERT P. RICCIARDI, Ph.D.**  
**PURSUANT TO 37 C.F.R. §1.132**

I, Robert P. Ricciardi declare as follows:

1. I am an inventor in the above-identified patent application.
2. I received my Ph.D. degree in Cellular and Molecular Biology from the University of Illinois in 1977. I have over 30 years of experience in this field, which is the subject of this application. I have read the outstanding Office Action and understand the Examiner's position.
3. I understand that the Examiner rejects my claims as indefinite, and rejects my application as lacking sufficient description and enablement. My Declaration addresses each individual basis of the Examiner's rejections.
4. My claimed method, as currently amended, reads as follows; I have underlined the terms the Examiner deemed unclear:

A method comprising

assessing a relative degree to which a human is susceptible to an undesirable bone density condition by identifying a polymorphic form identified as associated with a bone density pathology in each of

a gene encoding a vitamin D receptor present in the human's genome, and

a gene encoding interleukin-6 present in the human's genome,

thereafter calculating a susceptibility value for the condition by either

summing the identified polymorphisms to yield a value for the human, or

assigning a weighting factor to each polymorphism and then summing the weighting factors to yield a value for the human,

wherein a value for the human greater than a value for a control indicates a greater susceptibility to the undesirable bone density condition for the human, and wherein the polymorphic form identified as associated with any bone density pathology is a disorder associated polymorphism,

the method hereby assessing the relative degree to which the human is susceptible to the undesirable bone density condition.

The Examiner states that "it is unclear what encompasses calculating a susceptibility value for the condition by either summing the identified polymorphisms to yield a value for the human, or assigning a weighting factor to each polymorphism and then summing the weighting factors to yield a value for the human". I disagree, because using available information or information obtained without undue experimentation, one of ordinary skill in the art can calculate a susceptibility value for these polymorphisms. For any polymorphisms that do not have a known value, my method assigns a value of 1.0. I now describe each of these steps in further detail.

5. I disagree that summing the identified polymorphisms to yield a value for the human is unclear.

6. As I disclose in my application, the mere presence of a polymorphism in a gene encoding a Vitamin D receptor and a gene encoding interleukin-6 (IL-6) has predictive value to one of ordinary skill in the art. Even if no additional information is known about it, such as exactly how it correlates to a bone density condition, as long as it is present, it is assigned a value of 1.0. These assignments occur for each polymorphism in each of the Vitamin D receptor and IL-6 genes. Also, as an example of my method, see Appendix A which provides data and analysis of an individuals' susceptibility to oxidative stress.

7. The values are then summed (i.e., added). The summed value is a value for the human.

8. I disagree that assigning a weighting factor for each polymorphism and then summing the weighting factors to yield a value for the human is unclear.

9. Some polymorphisms are known to one of ordinary skill in the art as highly associated with a bone density condition. The presence of such polymorphisms, therefore, has greater predictive value. To account for the increased predictive value of these highly associated polymorphisms,

my method assigns a weighting factor. The weighting factors for each polymorphism present in each of the Vitamin D gene and the IL-6 gene are then summed to yield a susceptibility value.

10. My application discloses how to assign a weighting factor to each such highly predictive polymorphism.

$$\text{weighting factor} = (\text{correlation factor}) (\text{constant})$$

11. The "correlation factor" is determined by how closely the polymorphism correlates with the bone density condition, as known to one of ordinary skill in the art. For example, a human that is homozygous for the polymorphism will have a higher correlation factor than a human that is heterozygous for the polymorphism (e.g. ¶16).

12. The "constant" is determined by how relevant the polymorphism is to the bone density condition, as known to one of ordinary skill in the art. As I disclose at ¶16, one way to determine the "constant" is by an "odds ratio" (also known as "relative risk").

$$\text{odds ratio} = \frac{\text{odds of a bone density condition occurring in one group}}{\text{odds of the same bone density condition occurring in another group}}$$

The groups compared are humans who (a) lack the polymorphic allele, (b) are heterozygous for the polymorphic allele, or (c) are homozygous for the polymorphic allele. Thus, the "correlation factor" and "constant" are derived from data known by or available to one of ordinary skill in the art.

13. The weighting factor is then assigned. As one example, Gennari et al., which I cite, demonstrates how my method may be used. Gennari correlates a polymorphism in the vitamin D receptor gene with osteoporosis, a known bone density condition. Specifically, he finds a statistically significant two-fold increase in the prevalence of a T/C polymorphism in the initiation codon of the human vitamin D receptor gene in patients with osteoporosis, compared to patients without osteoporosis (p. 1382). Information such as this also provides an example of how to calculate my claimed "correlation factor". For example, see Gennari Table 4 where

$$\begin{aligned} \text{number of homozygous patients exhibiting a bone density disorder} &= 44 \\ \text{total number of homozygous patients} &= 55 \\ \text{"correlations factor"} &= 44/55 = 0.80 \end{aligned}$$

My claimed "constant" is calculated using the relevance between the polymorphic allele and a bone density condition, as shown in Table 5. For example, using the presence of vertebral fractures in patients, which indicates an undesirable bone density condition, 25% of patients having vertebral fractures were homozygous for the bone density disorder-associated polymorphism. Conversely, only 11% of patients not having vertebral fractures were homozygous for the bone density disorder-associated polymorphism.

14. Using this information, Gennari reports an odds ratio of 2.58 for homozygous patients and 1.59 for heterozygous patients. A person of ordinary skill in the art calculates or obtains the "correlation factor" and the "constant", then calculates the weighting factor as follows:

$$\begin{aligned}\text{weighting factor of homozygous polymorphism} &= (\text{correlation factor}) (\text{constant}) \\ &= (0.80) (2.58) \\ &= 2.1\end{aligned}$$

In my method, the weighting factor is determined for each polymorphism in each of the vitamin D receptor gene and the IL-6 gene in the individual.

15. All the weighting factors are summed to "yield a value for the human" which "assess his/her relative degree to which he/she "is susceptible to an undesirable bone density condition", as I claim.

16. I disagree with the Examiner that the metes and bounds of the control are unclear in my claims that a value for the human greater than a value for a control indicates a greater susceptibility to the undesirable bone density condition for the human.

17. A value for a control can be derived from either an individual other than the test individual, or in a hypothetical individual. For example, the susceptibility value in a hypothetical individual can be assumed to be 0. Thus, for each polymorphism in each of a gene encoding a vitamin D receptor and a gene encoding IL-6 in the test individual, the individual's susceptibility to a bone density condition increases relative to the hypothetical individual. In this way, even if the correlation factor and constant are not known for a particular polymorphism, the presence of the polymorphism alone can access susceptibility to a bone density condition in each of the vitamin D receptor gene and the IL-6 gene.

18. I disagree that the oligonucleotides encompassing a disorder associated polymorphism and non-disorder associated polymorphism are unclear.

19. My claimed method requires

assessing the occurrence of an individual disorder associated polymorphism by contacting a nucleic acid derived from the human's genome with a first oligonucleotide that anneals with higher stringency with the disorder associated polymorphism than with a corresponding non-disorder associated polymorphism, and

assessing annealing of the first oligonucleotide and the nucleic acid,

whereby annealing of the first oligonucleotide and the nucleic acid indicates that the human's genome comprises the disorder associated polymorphism.

20. One of ordinary skill in the art knows that oligonucleotide annealing is a specific assay. A first oligonucleotide specific for a bone density associated polymorphism in each of a vitamin D receptor gene and an IL-6 gene is known to anneal with higher stringency to these polymorphisms than to other sequences. Therefore, one of ordinary skill in the art would know the metes and bounds of a first oligonucleotide that anneals with higher stringency with the disorder associated polymorphism than with a corresponding non-disorder associated polymorphism, as my claim requires.

21. Assessing the relative degree to which a human is susceptible to an undesirable bone density condition, as I claim, requires determining whether a polymorphism in each of the vitamin D receptor and the IL-6 gene is present. Their presence is assessed by contacting a nucleic acid derived from the human's genome with a first oligonucleotide that anneals with higher stringency with the disorder associated polymorphism than with a corresponding non-disorder associated polymorphism, as my claim requires. A second oligonucleotide that anneals with higher stringency with a non-disorder associated polymorphism than with the corresponding disorder associated polymorphism may also be used.

22. For an example of oligonucleotides encompassing disorder associated polymorphism and non-disorder associated polymorphisms, continuing to use Gennari's example, a first oligonucleotide would preferentially bind to the polymorphic sequence containing T, and a second oligonucleotide would preferentially bind to the polymorphic sequence containing C. Using this method, the presence of the polymorphism is detected, as well as whether the polymorphism is homozygous or heterozygous, i.e., whether 0, 1, or 2 of the alleles contains the disorder associated polymorphism. Determining homozygosity of a bone density associated polymorphism "assesses the relative degree to which the human is susceptible to the undesirable bone density condition", as I claim.

23. I disagree that my application "fails to disclose first and second oligonucleotides especially in context of any and all variants gene encoding vitamin D receptor and interleukin-6". One of ordinary skill in the art recognizes that, using known polymorphisms identified as associated with a bone density pathology in each of the vitamin D receptor gene and IL-6 gene, he/she can create substantially complimentary oligonucleotides to the polymorphic forms of each of these genes, as I subsequently explain.

24. One of ordinary skill in the art knows or can determine sequences of gene variants of each of the vitamin D receptor gene and IL-6 gene. For example, publicly available searchable databases such as Genbank, SwissProt, and GenCards provide this information, as well as polymorphisms in each of the vitamin D receptor gene and IL-6 genes. Publicly available searchable databases such as HapMap Project, dbSNP, SNP500 Cancer, GeneSNPs, Seattle SNPs, regulatory-rSNP, and SNPtagger also provide polymorphisms. As only one example, the vitamin D receptor page on GenCards (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=VDR&search=vitamin+D+receptor&suff=txt>) displays known single nucleotide polymorphisms (SNPs). Association of these SNPs with bone density associated disorders can also be found in databases such as OMIM (Online Mendelian Inheritance in Man), AKS (AlmaKnowledgeServer), and PharmGKB. These sites show vitamin D receptor SNPs associated with osteoporosis and bone resorption, along with the sequences and related scientific reference. Thus one of ordinary skill in the art can readily determine, without undue experimentation, the sequences of each of the vitamin D receptor gene and the IL-6 gene, as well the sequences of the polymorphisms associated with each of these genes. Thus, one of ordinary skill in the art can use these data to generate a first and second oligonucleotide, as my claim requires.

25. I disagree with the Examiner that "demonstration of an association between a candidate gene [polymorphism] and [a bone density related disorder] does not necessarily mean that the gene [polymorphism] is causally responsible for the effect observed".

26. I claim that the susceptibility value allows assessing the relative degree to which the human is susceptible to the undesirable bone density condition. One of ordinary skill in the art recognizes that the problems with "confounding factors" and "population stratification" are largely negated by techniques known in the art. For example, Ferrari et al., which I cite in my application, addresses potential problems in the association between IL-6 polymorphisms and bone density associated disorders by evaluating data using Kruskal-Wallis analysis, analysis of variance (ANOVA), and standard linear regressions. Confounding factors (e.g., dietary and clinical variables) are also evaluated using, e.g., logistic regression at a 95% confidence interval. Thus, the association between the polymorphism and the bone density condition disorder can be established.

27. I disagree that my application "fails to provide any evidence, which establishes that assessment of these SNPs in combination would be a better predictor of assessing an undesirable bone density [disorder] as compared to identification of a single SNP". My claims

require assessing a human's susceptibility to an undesirable bone density condition by identifying a polymorphic form in each of a vitamin D receptor gene and an IL-6 gene.

28. One of ordinary skill in the art recognizes that undesirable bone density conditions (using osteoporosis as only one example) are multi-genetic pathologies (e.g., undesirable bone density conditions implicate other genes, such as type I collagen gene and estrogen receptor gene as well as the vitamin D receptor gene and the IL-6 gene). Therefore, assessing the presence of multiple SNPs better assess the susceptibility of an individual to undesirable bone density conditions.

29. I understand the Examiner questioned how my method controls for variation found in various sub-populations, e.g., ethnic groups.

30. My method does not need to account for polymorphism frequencies in various sub-populations. In some cases, certain populations, e.g. ethnic populations, exhibit polymorphic allelic frequencies that are different from a general population. However, the presence of one of my claimed genetic polymorphisms indicates an increased susceptibility to an undesirable bone density condition. This increased susceptibility occurs whether the polymorphism is rare or common in a particular group, because my method assesses the relative susceptibility of a human to an undesirable bone density condition, not the frequency or probability that that human will develop a bone density condition. For example, ethnic group A has a very low frequency of bone density associated polymorphism P. If a human which belongs to ethnic group A exhibits polymorphism P, then my method indicates that this human is relatively more susceptible to a bone density pathology. The frequency of the polymorphism in a population is only important in determining the absolute probability that that individual will develop a bone density related disorder.

31. I understand the Examiner questioned how my method would account for SNPs that increase, versus decrease, activity or expression of the encoded protein.

32. SNPs that increase activity or expression of the encoded protein are very rare. My method accounts for the rare occurrence of polymorphisms that result in an increase in activity or expression of the encoded protein. As amended claim 65 recites, the method is accomplished by "... identifying a polymorphic form identified as associated with a bone density pathology ...". Thus, it is the presence of the polymorphic form associated with a bone density pathology that is used to assess the individuals susceptibility. In the case where a polymorphism increases activity or expression of the encoded protein, then the polymorphic form is not associated with the bone

density pathology. In this case, the non-polymorphic form of the gene would be the form that is associated with a bone density pathology and therefore used in assessing susceptibility. For example, a polymorphism that is defined by allele "A" and allele "a" exhibits two different expression levels or activities for the protein encoded by the gene containing polymorphisms "A" and "a". Whether "A" has greater activity or expression than "a", or vice versa, is inconsequential. My method requires the identification of the polymorphic form that is associated with a bone density pathology, and then uses the presence of that polymorphism to assess susceptibility.

33. For at least the above reasons, I believe my Declaration demonstrates that the claims are sufficiently clear and that my application sufficiently describes and enables my claimed method.



I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both , under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the subject application or any patent issued thereon.

November 15, 2007

Date

Robert P. Ricciardi

Robert P. Ricciardi, Ph.D.

664137

## Appendix A – Proof of Concept

### "Skin-Genes Profile Test of 177 Women"

**Summary** – A Gene-SNP skin profile of 177 women demonstrated that this test will be useful. The test highlights the high frequency of Gene-SNP markers for skin wrinkling, detoxification, and oxidative damage. By providing different levels of support, this Gene-SNP profile enables us to offer personalized skin care formulations that will act to help slow down or help prevent various types of damage to the skin.

**The Process** – Genelink collected DNA samples, via buccal swabs, from 177 individuals. The samples were genotyped and screened for SNPs in genes associated with Oxidative Stress. The frequencies of the SNPs in the test population are shown in Table 1. The Gene-SNP test data is used to create a profile describing an individuals relative susceptibility to various skin health problems and allows customization or personalization of products for each individual. Specifically, the Gene-SNP test recommends skin health areas where test individuals need additional support based on their particular susceptibility value.

**Table 1. Gene SNP Frequencies (%)**

number of participants = 177

Recommended Skin-Care Support				
Skin SNPs	Functional Area	Maximum	Added	Basic
		Red	Yellow	Green
MSOD (OS1)	Oxidative Stress	2%	29%	69%
GPX1 (OS2)	Oxidative Stress	1%	12%	88%

#### Interpreting Test Results

A GREEN test result (Homozygous Negative) predicts that you do not have any variant SNPs and that the protein molecules expressed by this given gene are functioning optimally to produce its specific enzyme, hormone, cytokine, or structural protein. A comprehensive BASIC SUPPORT skin-care formulation is recommended to keep this system functioning optimally.

A YELLOW test result (Heterozygous Positive) indicates that you have one variant SNP and that a portion of the protein molecules expressed by this given gene, are functioning suboptimally to produce a specific enzyme, hormone, cytokine or structural protein. In order to compensate for the less than optimal function of the protein molecule, ADDED skin-care support for this area is recommended.

A RED test result (Heterozygous Positive) indicates that you have two variant SNPs and that the protein molecule expressing a specific enzyme, hormone, cytokine or structural protein is functioning minimally. As a result, it is important to have a MAXIMUM SUPPORT skincare formulation in place to compensate for this inferior protein molecule.

Sample_Id	hSD2_V16A	hGPX1	Sample_Id	
070300011936	1 2	1 2	070300011936	
070300012106			070300012106	
070300011983			070300011983	
070300011935			070300011935	
070300012087			070300012087	
070300012077			070300012077	
070300011938			070300011938	
070300012005		1 2	070300012005	
070300011964			070300011964	
070300011966			070300011966	
070300011967			070300011967	1 2
070300012027			070300012027	
070300012028			070300012028	
070300012022			070300012022	
070300012026	1 2		070300012026	
070300012031	1 2		070300012031	
070300012023			070300012023	1 2
070300012053	1 2		070300012053	1 2
070300012056		1 2	070300012056	1 2
070300012059			070300012059	1 2
070300012060			070300012060	1 2
070300012058			070300012058	1 2
070300011941		1 2	070300011941	
070300012038	1 2		070300012038	1 2
070300011971	1 2		070300011971	1 2
070300011978			070300011978	
070300012011	1 2		070300012011	
070300011920			070300011920	1 2
070300011955		1 2	070300011955	
070300012091			070300012091	1 2
070300011993	1 2		070300011993	1 2
070300011983		1 2	070300011983	1 2
070300011975	1 2		070300011975	
070300011918			070300011918	
070300011919	1 2		070300011919	
070300012089			070300012089	
070300011974			070300011974	

A "1" indicates the presence of a disorder associated polymorphism and a "2" indicates the presence of a non-disorder associated polymorphism. The two numbers represent each allele of the gene. The hSD2\_V16A and hGPX1 genes are both associated with oxidative stress. The data shows for example, individual 11936 is more susceptible to oxidative damage than, for instance, individuals 12005 and 11964 which exhibit one and zero, respectively, of disorder associated polymorphisms in at least two genes associated with oxidative damage.

Conclusion – the Gene-SNP test is an excellent predictor for learning in advance the functional areas of skin health that need 'customized' care as a means to prevent future skin damage and wrinkling. By providing different recommended levels of support, this Gene-SNP profile enables personalized skin-care formulations that will act to help slow-down or help prevent various types of damage to the skin.

## Vitamin D and Estrogen Receptor Allelic Variants in Italian Postmenopausal Women: Evidence of Multiple Gene Contribution to Bone Mineral Density

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### ABSTRACT

Bone mass and bone turnover are under genetic control. Restriction fragment length polymorphisms (RFLPs) at the vitamin D receptor (VDR) gene locus have been recently correlated to bone mineral density (BMD) and rate of bone loss. However, agreement on this relationship is not universal. The existence of ethnic and environmental differences between populations, a health-based selection bias in several previous studies, and the involvement of other genes could explain these discordant findings. In this study, we examined the relationship of VDR and estrogen receptor (ER) gene RFLPs with lumbar spine and upper femur BMD in 498 Italian postmenopausal women, 57.7 ± 0.4 yr old (144 normal, 106 osteopenic, and 176 osteoporotic). VDR gene RFLPs for *Apal*, *Bsm I*, and *TaqI* restriction endonucleases and ER RFLPs for *PvuII* and *XbaI* restriction endonucleases were assessed by Southern blotting analysis and were indicated, respectively, as A-a, B-b, T-t, P-p, and X-x (uppercase letters signifying the absence and lowercase letters the presence of the restriction site). After correcting for potential confounding factors (age, height, weight, age since menopause, osteophytosis, and facet joint osteoarthritis), a statistically significant VDR genotype effect on lum-

bar BMD ( $P = 0.01$ , analysis of covariance), but not on femoral BMD, was detected, with subjects in AABbtt genotype showing a 13% lower BMD than those with aabBtT genotype ( $P < 0.05$ , Tukey's test). Moreover, a statistically significant prevalence of AABbtt genotype in osteoporotics, and of AabTtT and aabTtT genotypes in nonosteoporotics, were detected. Conversely, there was no significant relationship of ER genotype to either lumbar or femoral BMD, even though a trend for higher BMD values in women with the ER PP genotype (with respect to those with ER pp genotype) was detected. When mean lumbar BMD was calculated for women grouped by ER and VDR genotype, we observed a significant difference between those within the 2 opposite associations AABbtt-PPXX and aabTtT-ppxx ( $0.71 \pm 0.05$  vs.  $0.97 \pm 0.03$  g/cm<sup>2</sup>,  $P < 0.05$  Tukey's test). These results are consistent with a segregation of the VDR AABbtt genotype with a higher risk of developing osteoporosis, in the Italian female population. The introduction of another variable, the ER genotype, in the analysis of VDR genetic determination of BMD, may represent a useful model in the identification of patients at risk of developing a multigenic disorder like osteoporosis. (*J Clin Endocrinol Metab* 88: 939–944, 1998)

**B**ONE mineral density (BMD), the major determinant of osteoporotic fracture risk, is a quantitative trait determined by the interaction of genetic, metabolic, and environmental factors. In the last years, both twin and family studies have suggested a major genetic contribution in bone mass determination and in the development of osteoporosis (1–4). The number of candidate genes is large, ranging from those regulating calcium homeostasis to the several locally involved in bone cell recruitment and activity. The vitamin D receptor (VDR) gene has been shown to be a major locus for genetic influences on bone mass, and polymorphisms in this gene seemed to predict spinal and femoral BMD in an Australian population (5). The subjects homozygous for the presence of the *Bsm I* restriction endonuclease site were reported to have a bone mass about 15% higher than that of subjects homozygous for the absence of the site (5). Successively, haplotype analysis, based on three restriction fragment

length polymorphisms (RFLPs) at the VDR gene locus, showed the possibility to discriminate individuals at high and very low osteoporotic risk, with subjects homozygous for the abT haplotype showing lumbar BMD values 0.15 g/cm<sup>2</sup> greater than subjects homozygous for the AbT haplotype (6). However, agreement on this relationship is not universal among different populations, some finding positive associations (7–12), others reporting no significant effect (13–17).

The cause of the discrepancies remains to be determined, and in part, it may be caused by the limited sample size of many studies. Indeed, relationships between VDR gene alleles and osteoporotic risk, mediated through differences in BMD, are unlikely to be observed in relatively small samples. A potential confounder in all such studies may be given also from the health-based selection bias, with the tendency to exclude osteoporotic women. Currently, the few association studies, in which the prevalence of VDR genotypes in osteoporotic and nonosteoporotic patients are compared, have been carried out in small samples, with limited statistical power (12, 15, 16, 18). Heterogeneity also is likely, with different major genes segregating in different patient sam-

Received April 8, 1997. Revision received July 28, 1997. Rerevision received October 21, 1997. Accepted November 11, 1997.

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ples. In this regard, other polymorphic genes, such as those encoding for estrogen receptor (ER), collagen type I  $\alpha 1$ , and interleukin 6 recently have been linked to variation in BMD (19–21). These genes could either positively or negatively modulate VDR gene effect, with a different power, which needs to be individually evaluated. In addition, environmental factors also could reciprocally interact with genetic factors. Recently, dietary calcium intake has been reported to contribute to the expression of the VDR gene effect, both on BMD (10, 11) and on intestinal calcium absorption (22). Finally, linkage disequilibrium with another bone metabolism-related gene on chromosome 12 (i.e. Collagen 2 A1 and retinoic acid receptor genes) cannot be excluded.

Being aware of the constant interaction between genetic and environmental factors in bone mass determination, the present study was performed to evaluate, for the first time, in a large and ethnically homogeneous group of postmenopausal women of Italian descent (stratified for BMD in normal, osteopenic, and osteoporotic patients): 1) the relationship of VDR and ER gene polymorphisms with BMD, after controlling for multiple confounders; and 2) the possibility of an interaction between VDR and ER genotypes with bone mass, assuming that variation of BMD is influenced by multiple genes.

### Subjects and Methods

#### Subjects

Patients eligible for the study were selected among 1500 consecutive postmenopausal women who, in 1995, attended the metabolic bone diseases outpatient clinics in Siena and Florence for osteoporotic risk evaluation. For all subjects, a detailed medical history was obtained, and dietary calcium intake was assessed by a sequential self-questionnaire, including foods that account for the majority of calcium in the diet. Among this group of women, 866 had associated conditions known to affect bone metabolism and were excluded from analysis. These were diseases known to influence bone mass (98 women), use of bone active drugs (303 estrogen replacement therapy, 198 vitamin D metabolite, 96 bisphosphonate, 59 calcitonin, and 7 fluoride) or use of drugs that could potentially affect bone metabolism (55 glucocorticoid, 34 thyroid hormone, and 16 antacid). Eighty-nine women also were excluded because of their different ethnic origin. Blood was available for DNA isolation in 426 of the remaining 545 subjects. The age range of the studied women was 47–76 yr, with a mean ( $\pm$ SEM) age of  $57.7 \pm 0.43$  yr. On the basis of BMD measurements and according to WHO criteria (23), 40% of the 426 subjects had osteoporosis, 26% had osteopenia, and 34% were normal. General characteristics of the population are presented in Table 1.

#### Genotyping

Genomic DNA was isolated from EDTA blood samples by a standard phenol-chloroform extraction procedure, and 8  $\mu$ g of DNA were digested for 6 h in a vol of 50  $\mu$ L with, respectively, 40 U of *Apa*I, *Bsm* I, *Taq*I, *Pvu*II, and *Xba*I restriction endonucleases (Boehringer Mannheim, Milan, Italy) at temperatures recommended by the manufacturer. The digested DNA was size-fractionated using 0.7–1% agarose gel electrophoresis and transferred to nylon-based filters (GeneScreen Plus, NEN Research Products, Boston, MA) by standard techniques (24). Filter membranes were prehybridized, then hybridized with the  $^{32}$ P-labeled probe for BMD at 65°C, washed, and autoradiographed for 24–48 h at –80°C in intensifying screen. For identifying the RFLPs, we used, as probes, a 2.1-kilobase complementary DNA (cDNA) coding region of the human VDR (25) and a 1.8-kilobase cDNA coding region of the human ER (26). The probes were radiolabeled with [ $\alpha$ - $^{32}$ P]deoxycytidine triphosphate using a random priming labeling kit (Boehringer Mannheim, Milan, Italy). The RFLPs were coded as B-b (*Bsm* I), A-a (*Apa*I), T-t (*Taq*I), P-p (*Pvu*II), and X-x (*Xba*I), uppercase letters signifying the absence and lowercase letters the presence of the restriction site.

#### Bone densitometry

Lumbar BMD (L2–L4), measured by dual-energy x-ray absorptiometry (Hologic QDR 1000/W) was available for all the 426 studied women. The long term *in vitro* precision at this site measured on spinal phantom was 0.4% in Siena and 0.6% in Florence; the *in vivo* precision was 0.9% in both centers. Dual-energy x-ray absorptiometry BMD scans at the upper femur were available for 230 of the 426 women (113 osteoporotic, 58 osteopenic, and 59 normal), with *in vivo* coefficients of variations of 1.1% in Florence and 0.9% in Siena. Cross-calibration studies on the precision of measurements between the 2 centers were previously performed both *in vitro* (using a single anthropomorphic lumbar spine phantom) and *in vivo*, on 50 patients, covering most of the clinically observed spinal density range. A correction factor was not considered necessary.

Because of the influence of extravertebral calcification on spinal bone mass measures, each woman underwent a lateral lumbar spine x-ray examination to be scored for spinal osteophytosis (SPO), according to Orwoll (27), and for facet joint osteoarthritis (FOA) on a four-point scale (0 = none, 1 = mild, 2 = moderate, and 3 = severe), according to Masud (28). Vascular calcifications were not evaluated for their reported limited impact on spinal density measurements (27–29).

#### Statistical analysis

Data were expressed as mean  $\pm$  SEM, with  $P < 0.05$  accepted as the value of significance. The frequency distribution of VDR genotypes in osteoporotic, osteopenic, and normal groups were compared using cross-tabulation and standard  $\chi^2$  tests. Differences in anthropometric characteristic, spinal BMD, and femoral BMD among the different VDR and ER genotypes were tested using ANOVA. Similar comparisons were done after adjusting mean BMD values for potential confounding factors such as age, height, weight, years since menopause (YSM), SPO, and

TABLE 1. General characteristics of the study group patients

	Normal	Osteopenic	Osteoporotic	Total
n	144	106	176	426
Age (yr)	$57.1 \pm 0.7$	$57.9 \pm 0.8$	$58.2 \pm 0.6$	$57.7 \pm 0.4$
YSM	$8.5 \pm 0.9$	$9.0 \pm 0.9$	$10.1 \pm 0.7$	$9.3 \pm 0.5$
Height (cm)	$163.9 \pm 0.8^a$	$161.6 \pm 0.7$	$159.4 \pm 0.6$	$161.4 \pm 0.4$
Weight (kg)	$64.1 \pm 1.3$	$64.5 \pm 1.2$	$62.9 \pm 1.2$	$63.7 \pm 0.6$
Ca intake (mg/day)	$545 \pm 142$	$568 \pm 164$	$587 \pm 128$	$567 \pm 102$
Lumbar BMD (g/cm $^2$ )	$1.05 \pm 0.01^a$	$0.88 \pm 0.01^a$	$0.69 \pm 0.01$	$0.86 \pm 0.01$
SPO score	$0.79 \pm 0.07$	$0.73 \pm 0.09$	$0.63 \pm 0.10$	$0.70 \pm 0.05$
FOA score	$0.63 \pm 0.06$	$0.57 \pm 0.08$	$0.57 \pm 0.09$	$0.58 \pm 0.04$
Corrected BMD (g/cm $^2$ ) <sup>b</sup>	$1.03 \pm 0.01^a$	$0.85 \pm 0.01^a$	$0.67 \pm 0.01$	$0.83 \pm 0.01$

Values are expressed as mean  $\pm$  SEM.

<sup>a</sup>  $P < 0.05$  ANOVA vs. osteoporotic group.

<sup>b</sup> Lumbar spine BMD values, corrected for age, height, weight, YSM, SPO, and FOA scores.

FOA scores, using analysis of covariance. Between groups, differences among genotype groups were tested using Tukey's test. All statistical analyses were performed using STATGRAPHICS (Manugistic Inc., Rockville, Maryland) and Statistica 5.1 (Statsoft Inc., Tulsa, Oklahoma).

## Results

### VDR RFLPs

No significant departures from Hardy-Weinberg equilibrium were observed for *Apal*, *Bsm I*, and *TaqI* RFLPs. The distribution of genotypes was similar to that of other studies, in Caucasians. When the 3 RFLPs at the VDR gene locus were considered together in all subjects analyzed, 7 major genotypes were recognized: AaBbTt (n = 157), AABbTt (n = 71), aabbTT (n = 56), AABbTt (n = 49), AabbTT (n = 47), AAbbTT (n = 17), and AaBbTT (n = 13).

Clinical characteristics of patients, in relation to the VDR genotype, are shown in Table 2. Sixteen women having rare genotypes (n < 5) were excluded from analysis. Results indicated that subjects in the seven most common genotypes were well matched for age and did not significantly differ for YSM, height, weight, and dietary calcium intake. After correcting for potential confounding factors, a statistically significant segregation of VDR genotypes with lumbar BMD was detected (P = 0.01, analysis of covariance), with mean corrected BMD values at the lumbar spine significantly higher in women with aabbTT genotype, compared with those with either AABbTt or AaBbTt genotype (P < 0.05, Tukey's test). A similar, but not significant, trend was observed for femoral neck BMD, with a 6% higher BMD value in aabbTT than in AABbTt genotype (data not shown).

When *Bsm I* and *TaqI* polymorphisms were analyzed separately, we observed a significantly increased lumbar BMD in women with the bb or the TT genotypes than in those with BB and tt genotypes, respectively (0.87 ± 0.01 vs. 0.78 ± 0.02, bb vs. BB, P < 0.05, Tukey's test; and 0.86 ± 0.01 vs. 0.78 ± 0.02, TT vs. tt, P < 0.05, Tukey's test). Conversely, no differences in mean BMD levels for different *Apal* genotypes were detected (data not shown).

Genotype determinations for osteoporotic, osteopenic, and normal groups are summarized in Table 3. We observed a significantly increased prevalence of AABbTt genotype in osteoporotic and osteopenic patients, compared with non-osteoporotic ( $\chi^2 = 14.7$ ; P = 0.0006). On the contrary, aabbTT

and AabbTT genotypes were significantly overrepresented in nonosteoporotic vs. osteoporotic women ( $\chi^2 = 8.75$ , df = 1, P = 0.003 for aabbTT genotype; and  $\chi^2 = 6.05$ , df = 1, P = 0.04 for AabbTT genotype). Cross-tabulation testing resulted statistically significant ( $\chi^2 = 41.9$ , df = 14, P = 0.0004).

### ER RFLPs

*PvuII* and *XbaI* RFLPs allele frequencies in this Italian population followed Hardy-Weinberg equilibrium. When we combined the 2 RFLPs we recognized six genotypes: PpXx (n = 174), ppXx (n = 126), PPXX (n = 73), PpXx (n = 25), PpXx (n = 17), and PpXx (n = 11). Three genotypes (PPxx, ppXX, and ppXx) were not detected in the population examined in this study.

The clinical characteristics, by ER genotype, of the 426 studied women are given in Table 4. There were no significant differences in age, weight, height, YSM, and dietary calcium intake across genotypes. Analysis of the ER genotypes, in relation to adjusted BMD values, did not reveal any significant effect.

Single RFLP analysis revealed a trend for a 0.05 g/cm<sup>2</sup> higher lumbar BMD value in women with pp genotype than in those with PP genotype (data not shown). On the contrary, women with the xx genotype showed a 0.02 g/cm<sup>2</sup> lower lumbar BMD than those with XX genotype (data not shown). However, none of these differences reached statistical significance.

$\chi^2$  analysis on the genotypic frequencies revealed no significant increased prevalence of any of the six ER genotypes

TABLE 3. VDR Genotypes for normal, osteopenic, and osteoporotic patients

Genotype	Patients			$\chi^2$	P value
	Normal	Osteopenic	Osteoporotic		
AABbTt	11 (7.6%)	29 (18.9%)	40 (23.8%)	14.7	0.0006
AaBbTt	15 (10.4%)	11 (10.4%)	23 (13.7%)	1.0	0.6
AaBbTt	56 (38.9%)	37 (34.9%)	64 (38.1%)	0.5	0.7
AabbTT	8 (5.6%)	4 (3.8%)	5 (3.0%)	1.3	0.5
AaBbTT	5 (3.5%)	3 (2.8%)	5 (3.0%)	0.1	0.9
AabbTT	23 (16%)	12 (11.3%)	12 (7.1%)	6.0	0.04
aabbTT	26 (18%)	19 (17.9%)	11 (6.5%)	11.3	0.003

TABLE 2. Clinical features of the women in relation to VDR genotype; 16 of the 426 women exhibiting rare genotypes (n < 5) were excluded from analysis

	Genotype						
	AABbTt	AaBbTt	AaBbTt	AaBbTT	AaBbTT	AaBbTT	aabbTT
n	71	49	157	17	13	47	56
Age	55.8 ± 1.0	58.9 ± 1.4	57.4 ± 0.7	59.0 ± 2.7	58.8 ± 2.7	56.8 ± 1.3	57.2 ± 1.1
YSM	8.3 ± 1.6	9.7 ± 1.6	9.6 ± 0.8	7.9 ± 3.0	10.5 ± 3.3	9.8 ± 2.2	9.0 ± 1.4
Height (cm)	160.6 ± 0.9	161.5 ± 1.2	160.4 ± 0.7	163.6 ± 2.6	163.8 ± 2.6	163.1 ± 1.2	162.3 ± 1.0
Weight (kg)	63.4 ± 1.5	64.6 ± 2.0	63.2 ± 1.3	62.6 ± 4.1	63.1 ± 4.2	63.6 ± 1.9	65.0 ± 1.7
Ca intake (mg/day)	588 ± 162	607 ± 186	565 ± 132	549 ± 221	578 ± 227	551 ± 196	560 ± 189
FOA score	0.54 ± 0.12	0.70 ± 0.14	0.60 ± 0.08	0.47 ± 0.22	0.74 ± 0.30	0.63 ± 0.14	0.65 ± 0.12
SPO score	0.76 ± 0.12	0.61 ± 0.15	0.63 ± 0.09	0.58 ± 0.21	0.81 ± 0.27	0.70 ± 0.15	0.75 ± 0.13
Lumbar BMD	0.81 ± 0.03*	0.84 ± 0.03	0.85 ± 0.02*	0.87 ± 0.06	0.83 ± 0.07	0.89 ± 0.03	0.91 ± 0.03
Corrected BMD <sup>b</sup>	0.78 ± 0.02*	0.85 ± 0.03	0.82 ± 0.01*	0.85 ± 0.06	0.80 ± 0.07	0.87 ± 0.03	0.89 ± 0.02

Values are expressed as mean ± SEM.

\* P < 0.05 Tukey's test vs. aabbTT genotype.

<sup>b</sup> Lumbar BMD values, corrected for age, height, weight, YSM, FOA, and SPO scores.

TABLE 4. Clinical features of the 426 women in relation to ER genotype

	Genotype					
	PPXX	PPXx	PpXX	PpXx	PpXX	ppxx
n	73	17	11	174	25	126
Age	56.5 ± 1.0	58.2 ± 1.9	59.0 ± 2.6	57.6 ± 0.7	59.0 ± 1.7	58.1 ± 0.8
YSM	8.1 ± 1.2	9.5 ± 2.3	10.3 ± 3.1	9.3 ± 0.8	10.2 ± 2.0	9.7 ± 1.0
Height (cm)	161.5 ± 1.1	161.2 ± 2.3	163.7 ± 2.9	162.4 ± 0.7	158.3 ± 1.8	160.4 ± 0.8
Weight (kg)	62.6 ± 1.7	60.5 ± 3.2	65.4 ± 4.1	63.7 ± 1.1	63.3 ± 2.9	64.7 ± 1.3
Ca intake (mg/day)	554 ± 168	593 ± 221	556 ± 202	566 ± 121	601 ± 198	574 ± 186
FOA score	0.58 ± 0.12	0.49 ± 0.24	0.80 ± 0.30	0.43 ± 0.10	0.91 ± 0.18	0.76 ± 0.12
SPO score	0.85 ± 0.13	0.85 ± 0.27	0.90 ± 0.32	0.59 ± 0.10	0.83 ± 0.20	0.69 ± 0.11
Lumbar BMD	0.856 ± 0.02	0.872 ± 0.04	0.890 ± 0.04	0.850 ± 0.01	0.861 ± 0.03	0.873 ± 0.01
Corrected BMD*	0.825 ± 0.02	0.844 ± 0.04	0.866 ± 0.06	0.821 ± 0.01	0.833 ± 0.05	0.855 ± 0.01

Values are expressed as mean ± SEM.

\* Lumbar spine BMD values, corrected for age, height, weight, YSM, SPO, and FOA scores.

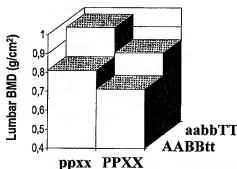


FIG. 1. Mean corrected lumbar BMD values, adjusted for potential confounding factors among women grouped by opposite VDR (AABBtt, aabbTT) and ER (PPXX, ppxx) genotypes.

in osteoporotic, osteopenic and nonosteoporotic groups (data not shown).

#### Combined effect of VDR and ER genes RFLPs

Sixteen major association groups were detected combining VDR and ER genotypes: AABBtt-PPXX (n = 9), AABBtt-PpXx (n = 29), AABBtt-ppxx (n = 25), AaBbTT-PPXX (n = 31), AaBbTT-PpXx (n = 70), AaBbTT-ppxx (n = 43), aabbTT-PPXX (n = 8), aabbTT-PpXx (n = 27), aabbTT-ppxx (n = 14), AaBbTt-PpXx (n = 10), AaBbTt-PPXX (n = 14), AaBbTt-ppxx (n = 12), AaBbTt-PpXx (n = 18), AaBbTt-PPXX (n = 12), AaBbTt-ppxx (n = 8), and AaBbTt-PpXx (n = 23).

As shown in Fig. 1, when mean adjusted lumbar BMD values were calculated among women grouped by ER and VDR genotypes, we observed a statistically significant difference of approximately 0.26 g/cm<sup>2</sup> between the two opposite association AABBtt-PPXX and aabbTT-ppxx (0.713 ± 0.05 vs. 0.970 ± 0.03 g/cm<sup>2</sup>; *P* < 0.05, Tukey's test). Furthermore, a trend for higher BMD values was detected between ppxx and PPXX subjects with the same VDR genotype (*P* = 0.08, AABBtt-PPXX vs. AABBtt-ppxx; *P* = 0.07, aabbTT-PPXX vs. aabbTT-ppxx).

#### Discussion

Evidence from epidemiological and twin studies clearly demonstrates that osteoporosis is a multifactorial disease with a strong genetic component. BMD, the major factor

determining bone strength and consequently osteoporotic fracture risk, can be considered a quantitative polygenic trait. Because the description that the genetic component responsible for bone mass could be largely ascribed to a simple allelic change in the VDR gene (5), several conflicting reports on the relationship between VDR genotypes and BMD have been published (6–18, 22, 30). The VDR gene, however, is only one of a large group of candidate genes, ranging from those encoding cytokines and growth factors involved in local control of bone metabolism to those encoding collagenic and noncollagenic matrix components and those encoding calcitropic hormones and their receptors. Therefore, even though the VDR gene could represent a major gene in the determination of bone mass, other intrinsic and environmental influences certainly interact with it, and these interactions need to be quantified, to understand more of the causal spectrum (31).

Present data from a large and ethnically homogeneous population showed a significant segregation of VDR genotypes with lumbar BMD, in the Italian population, as previously shown in Australian women (5–6). Women in the AABBtt genotype group showed a spinal BMD 13% less than those with aabbTT genotype. Such a difference persisted even after adjustment of BMD values for the potential influence of SPO and FOA. The magnitude of this effect between extreme homozygotes was approximately 0.1 g/cm<sup>2</sup>, slightly lower than that reported by Morrison *et al.* (5–6). A similar trend was observed for femoral neck BMD, but with no statistically significant difference between genotypes. How can we reconcile these evidences with the lack of segregation of VDR alleles with BMD in several patient samples (13–17)? One possible cause of discrepancy could be related to the relatively small samples of many other studies being not powerful enough to adequately assess the VDR gene allele effect on bone mass. This explanation does not apply to two recent studies that have looked specifically at the relationship between VDR genotypes and BMD in large samples of postmenopausal women (17, 30). The first study (30), of Uitterlinden and co-workers, reported a weak association between low bone mass and a particular VDR genotype, AaBbTt, which is different from the one reported to be associated with low BMD, by our and other groups (4–12). In the 426 analyzed Italian women, individuals with AaBbTt genotype showed BMD values that were interme-

diate, between those with aabbTT and AABbTT genotypes. However, Uitterlinden *et al.* limited their analysis to the femoral neck BMD, a site with predominantly cortical bone, whereas the genetic effect on bone mass seems to be stronger at sites with higher proportions of trabecular bone (2–5). In the second study (17), no significant relationships between VDR genotypes and BMD (measured at the spine, hip, and forearm) were detected in 268 French postmenopausal women, with frequency distribution of VDR genotypes quite different from that previously reported both by the same authors (14) and in other Caucasian populations of European ancestry (30, 32). Moreover, the contribution of VDR polymorphisms, both in Dutch and French studies, may have been masked by the relatively high calcium intake (17, 30). Indeed, environmental factors, such as calcium intake, are known to differ widely between populations and have been shown to contribute to the genetic influence of VDR genotypes on BMD (10–11), rates of bone loss (10–11), and intestinal calcium absorption (22). Consistent with the work of Dawson-Hughes *et al.* (22), we recently observed a significantly reduced fractional absorption capacity of strontium in a group of calcium-depleted women with AABbTT genotype, with respect to those with aabbTT genotype, suggesting a segregation of the VDR AABbTT genotype with a lower intestinal calcium absorption efficiency (32). For all these reasons, it is possible that the influence of VDR genotypes on bone metabolism could be observed only among populations with a relatively low calcium intake, as in the Italian population (33). Interestingly, in this sample of Italian postmenopausal women, we also observed a statistically significant increase in prevalence of both AABbTT genotype in the osteoporotic group and aabbTT and AabbTT genotypes in the nonosteoporotic group, in agreement with a possible segregation of the AbT homozygous haplotype with a higher risk of developing osteoporosis. Similarly, 2 other studies showed a disproportionate representation of the B allele in osteoporotic subjects, compared with a control group; but in both of them, these differences did not reach statistical significance (12, 18). By contrast, Looney and co-workers referred no large overrepresentation of the BB genotype in a group of North American severely osteoporotic women, compared with age-matched controls (15). However, all these previous studies were conducted in a limited number of subjects, with a consequent limited statistical power to test the hypothesis of a prevalence of a given genotype in osteoporotic subjects (12, 15, 16, 18).

The present study extends observations relating VDR genotypes and demonstrates that the addition of ER genotype to VDR genotype determination may provide a tool to identify, more precisely, individuals with a reduced bone mass. The magnitude of the effect of combining both VDR and ER genotype determination on lumbar BMD reaches approximately 2 sd and is significantly greater than that obtained from the analysis of the single ER or VDR gene effect. From this study, it also seems that the ER genotype PPXX confers some reduction in spinal BMD only when combined with VDR AABbTT genotype, without showing a segregation with BMD values by itself. Kobayashi *et al.* recently reported a statistically significant association of ER P<sub>x</sub> haplotype with a lower BMD in postmenopausal Japanese women, indepen-

dent of VDR genotype (19). It is possible that these differences are related to the relative differential distribution of VDR and ER genotypes between populations of European and Asiatic ancestry. In fact, the P<sub>xx</sub> genotype, representative of 8% of the Japanese population, was not detected in any of the 426 women examined in this study, whereas VDR genotype AABbTT, which in our Italian population is associated with the lowest BMD values, is extremely rare in the Japanese population (8, 16, 34). For this reason, a hypothetical segregation of BMD with polymorphisms at the ER gene locus could be more easily detectable in Asiatic women (where just 2 VDR genotypes, aabbTT and AabbTT, account for almost 80% of the total population) than in Caucasian populations of European ancestry, which exhibit high heterogeneity in VDR gene polymorphisms.

The epistatic effect between the ER and the VDR genes on BMD determination may be biologically fundamental, supporting a relevant role of the ER gene locus on BMD. Analysis of larger sample populations will make it possible to ascribe the ER gene locus either to the major gene family or to the polygenic aggregate, whose members are recognized among loci whose genetic effect is individually small. This approach, however, may be considered useful in future complex models of segregation analysis for osteoporotic risk.

In conclusion, in this homogeneous population of Italian postmenopausal women with a relatively low calcium intake, the allelic changes at the VDR gene locus are responsible for an important portion of the genetic component of spinal BMD. The results from the association analysis of ER and VDR genotypes effect on lumbar BMD suggest that the ER RFLPs could play a role, as well, exerting an additional contribution to bone mass determination. Other polymorphic genes and environmental factors could further modulate the expression of ER and VDR allelic effect on bone mass, making the picture as complicated as it is in nature.

### Acknowledgments

We are grateful to Dr. Carlo Gennari for the careful reading of the manuscript and for his continuing support and encouragement. We also thank Pasquale Imperiale for expert technical assistance in the statistical analyses.

### References

- Smith DM, Nance WE, Kang KW, Christian JC, Johnston CC. 1972 Genetic factors in determining bone mass. *J Clin Invest* 52:2800–2808.
- Hansen MA, Hassager C, Jensen SB, Christiansen C. 1992 Is heritability a risk factor for postmenopausal osteoporosis? *J Bone Miner Res* 7:1037–1043.
- Seeman E, Hopper JL, Bach LA, *et al.* 1989 Reduced bone mass in daughters of women with osteoporosis. *N Engl J Med* 320:554–558.
- Eisman JA, Kelly P, Morrison NA, *et al.* 1992 Genetic and environmental interaction on bone mass. In: Cohn DV, Gennari C, Tashjian AH, eds. *Calcium regulating hormones*. Amsterdam: Elsevier Science Publisher B.V.: 376–383.
- Morrison NA, Cheng JQ, Akifumi T, *et al.* 1994 Prediction of bone density from vitamin D receptor alleles. *Nature* 367:284–287.
- Morrison NA, Tokita A, Cheng JQ, Eisman JA. 1995 Allelic variation in the vitamin D receptor gene: major haplotypes in Caucasians. *Bone* 16:105S (Abstract 8).
- Spector TD, Keen RW, Arden NK, *et al.* 1995 Influence of vitamin D receptor genotype on bone mineral density in postmenopausal women: a twin study in Britain. *Br Med J* 310:1357–1360.
- Matsumura T, Ishii S, Tokita A, *et al.* 1995 Vitamin D receptors and bone mineral density. *Lancet* 345:1239–1240.
- Fleet JC, Harris SS, Wood RJ, Dawson-Hughes B. 1995 The BSM 1 vitamin D receptor restriction fragment length polymorphism (BB) predicts low bone density in premenopausal black and white women. *J Bone Miner Res* 10:985–990.



10. Ferraci S, Rizzoli R, Chevalley T, Slosman D, Eisman JA, Bonjour JP. 1995 Vitamin-D receptor gene polymorphisms and change in lumbar-spine bone mineral density. *Lancet* 345:423-424.
11. Krall EA, Parry P, Lichter JB, Dawson-Hughes B. 1995 Vitamin D receptor alleles and rates of bone loss: Influence of years since menopause and calcium intake. *J Bone Miner Res* 10:978-984.
12. Riggs BL, Nguyen TV, Melton LJ, et al. 1995 The contribution of vitamin D receptor gene alleles to the determination of bone mineral density in normal and osteoporotic women. *J Bone Miner Res* 10:991-996.
13. Hustmyer FG, Peacock M, Hul S, Johnston CC, Christian JC. 1994 Bone mineral density in relation to polymorphism at the vitamin D receptor gene locus. *J Clin Invest* 94:2130-2134.
14. Garnero P, Borel O, Sornay-Rendu E, Delmas PD. 1995 Vitamin D receptor gene polymorphisms do not predict bone turnover and bone mass in healthy premenopausal women. *J Bone Miner Res* 10:1263-1268.
15. Looney JE, Yoon HK, Fischer M, et al. 1995 Lack of a high prevalence of the BB vitamin D receptor genotype in severely osteoporotic women. *J Clin Endocrinol Metab* 80:2158-2162.
16. Lim SK, Park YS, Park JM, et al. 1995 Lack of association between vitamin D receptor genotypes and osteoporosis in Korean. *J Clin Endocrinol Metab* 80:3677-3681.
17. Garnero P, Borel P, Sornay-Rendu E, Arlot ME, Delmas PD. 1996 Vitamin D receptor gene polymorphisms are not related to bone turnover, rate of bone loss and bone mass in postmenopausal women: the OFELY study. *J Bone Miner Res* 11:827-834.
18. Melhus H, Kindmark A, Amer S, Wilen B, Lindh E, Ljunger S. 1994 Vitamin D receptor genotypes in osteoporosis. *Lancet* 344:949-950.
19. Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M, Oshima H. 1996 Association of bone mineral density with polymorphism of the estrogen receptor gene. *J Bone Miner Res* 11:306-311.
20. Grant SFA, Reid DM, Blake G, Herd R, Fogelman I, Ralston SH. 1996 Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I  $\alpha 1$  gene. *Nat Genet* 14:203-205.
21. Murray RE, Grant SFA, Reid DM, Ralston SH. 1996 Bone mineral density and polymorphisms of the interleukin 6 gene. In: Papapoulos SE, Lips P, Pols HAP, Johnston CC, Delmas PD, eds. *Osteoporosis*. Amsterdam: Elsevier Science; 167-170.
22. Dawson-Hughes B, Harris SS, Finerman S. 1995 Calcium absorption on high and low calcium intakes in relation to vitamin D receptor genotype. *J Clin Endocrinol Metab* 80:3657-3661.
23. Kanis JA, Melton LJ, Christiansen C, Johnston CC, Khaltaev N. 1994 The diagnosis of osteoporosis. *J Bone Miner Res* 9:1137-1141.
24. Southern EM. 1975 Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98:503.
25. Baker AR, McDonnell DP, Hughes M, et al. 1988 Cloning and expression of full length cDNA encoding human VDR. *Proc Natl Acad Sci USA* 85:3294-3298.
26. Green S, Walter P, Kumar V, et al. 1986 Human estrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* 320:134-139.
27. Orwoll ES, Oviatt SK, Mann T. 1990 The impact of osteophytic and vascular calcifications on vertebral mineral density measurements in men. *J Clin Endocrinol Metab* 70:1202-1207.
28. Masud T, Keen R, Nandra D, Jawed S, Doyle DV, Spector TD. 1996 Facet joint osteoarthritis and bone density measurements. In: Papapoulos SE, Lips P, Pols HAP, Johnston CC, Delmas PD, eds. *Osteoporosis*. Amsterdam: Elsevier Science; 167-170.
29. National Osteoporosis Foundation Scientific Advisory Board. 1989 Clinical indications for bone mass measurements. *J Bone Miner Res* [Suppl 2]41-28.
30. Utterlinden AG, Pols HAP, Burger H, et al. 1996 A large scale population-based study of the association of vitamin D receptor polymorphisms with bone mineral density. *J Bone Miner Res* 11:1241-1248.
31. Brandt ML, Bianchi ML, Eisman JA, et al. 1994 Genetics of osteoporosis. *Calcif Tissue Int* 55:161.
32. Gennari L, Becherin L, Masi L, et al. 1997 Vitamin D receptor genotypes and intestinal calcium absorption in postmenopausal women. *Calcif Tissue Int* 61:460-463.
33. Gennari C, Montagnani M. 1990 Role of nutrition in the pathogenesis of osteoporosis. In: Fabris F, Pernigotti L, Ferrario E, eds. *Sedentary life and nutrition*. New York: Raven Press Ltd; 109-118.
34. Beavan S, Prentice A, Yan L, Dibba B, Ralston S. 1996 Differences in vitamin D receptor genotype and geographical variation in osteoporosis. *Lancet* 348: 136-137.

## Erratum

In the announcement of drugs newly approved by the FDA on page 360 in the February issue of *The Journal of Clinical Endocrinology and Metabolism* [83(2):360], two entries were mistakenly conflated. The announcement should have read:

Repaglinide, trade name Prandin, made by Novonordisk.  
Sibutramine, trade name Meridia, made by Knoll.  
Raloxifene, trade name Evista, made by Lilly.

The Editors regret the confusion caused by this error.

## Cloning and expression of full-length cDNA encoding human vitamin D receptor

(1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>)

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Communicated by R. O. Slatyer, January 11, 1988 (received for review October 9, 1987)

**ABSTRACT** Complementary DNA clones encoding the human vitamin D receptor have been isolated from human intestine and T47D cell cDNA libraries. The nucleotide sequence of the 4605-base pair (bp) cDNA includes a noncoding leader sequence of 115 bp, a 1281-bp open reading frame, and 3209 bp of 3' noncoding sequence. Two polyadenylation signals, AATAAA, are present 25 and 70 bp upstream of the poly(A) tail, respectively. RNA blot hybridization indicates a single mRNA species of ~4600 bp. Transfection of the cloned sequences into COS-1 cells results in the production of a single receptor species indistinguishable from the native receptor. Sequence comparisons demonstrate that the vitamin D receptor belongs to the steroid-receptor gene family and is closest in size and sequence to another member of this family, the thyroid hormone receptor.

Vitamin D<sub>3</sub> receptors (VDR) mediate the action of their cognate ligand 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] by controlling the expression of hormone-sensitive genes (1). These receptor proteins are intracellular polypeptides of 50–60 kDa that specifically bind 1,25(OH)<sub>2</sub>D<sub>3</sub> and interact with target-cell nuclei to produce a variety of biologic effects (2, 3). Recently, monoclonal antibodies (mAbs) have been used to recover cDNAs encoding a portion of the chicken VDR mRNA (4). The present study describes the cloning and characterization of cDNAs encoding the human vitamin D receptor (hVDR).<sup>†</sup> Analysis of the deduced amino acid sequence of hVDR demonstrates that this protein belongs to the superfamily of trans-acting transcriptional regulatory factors defined by the steroid and thyroid hormone receptors (5–9). These receptors are characterized by a highly conserved DNA-binding domain rich in cysteine, lysine, and arginine residues and a carboxyl-terminal hydrophobic ligand-binding domain. The primary sequence of the hVDR exhibits this characteristic domain structure and shows that the VDR is more closely related to the thyroid hormone receptor than to the other receptors. Expression of the cloned cDNA in COS-1 cells demonstrates that the single protein produced is indistinguishable from the native receptor in both physical properties and affinity for 1,25(OH)<sub>2</sub>D<sub>3</sub>.

### MATERIALS AND METHODS

**Identification of hVDR cDNAs.** An oligo(dT)-primed  $\lambda$ gt10 cDNA library (10) made from 5  $\mu$ g of human jejunal poly(A)<sup>+</sup> mRNA was screened using a 310-base pair (bp) EcoRI-Sau3A fragment of cVDR (4). The filters were hybridized at 42°C overnight in the following solution: 5 $\times$  SSC (1 $\times$  SSC = 150 mM sodium chloride and 15 mM trisodium citrate) 5 $\times$

Denhardt's solution (11)/0.1% sodium dodecyl sulfate/salmon sperm DNA at 200  $\mu$ g/ml<sup>-1</sup>/50% (wt/vol) formamide containing 1 $\times$  10<sup>6</sup> cpm of nick-translated probe per ml (12). After being washed three times at 60°C in 0.5 $\times$  SSC to remove excess probe, the filters were exposed to X-ray film (Kodak X-Omat S) at -70°C with an intensifying screen. Hybridization-positive phage were isolated, and their inserts were subcloned into the EcoRI site of M13mp8.  $\lambda$ VDR1 was obtained in this fashion and subsequently used to screen an Okayama-Berg (13) T47D cDNA library (provided by G. Ringold, Stanford University), yielding clone VDR3, and a specifically primed  $\lambda$ gt10 T47D library yielding clone  $\lambda$ VDR2. The latter was made by substituting the oligonucleotide 5' ACACACCCACAGATCCGGGG 3' for oligo(dT) in the first strand reaction (underlined in Fig. 2).

**DNA Sequence Analysis.** Three overlapping clones were used to generate the full-length VDR sequence cDNA inserts to be sequenced. These clones were subcloned into the EcoRI site of M13mp8 for sequencing by the dideoxynucleotide chain-termination method (14). Primers were either the M13 universal primer or sequence-derived oligonucleotides.

**RNA Blot Hybridization.** Total RNA was isolated from each of three cell lines (15), and the mRNA fraction was selected by successive passages over oligo(dT)-cellulose (16). The mRNA samples (10  $\mu$ g) were resolved on a 1% formaldehyde-agarose gel (17) and then transferred electrophoretically to a nylon membrane (Nytan; Schleicher & Schuell). The filter was hybridized to nick-translated hVDR-1 (1 $\times$  10<sup>6</sup> cpm/ $\mu$ g; 1 $\times$  10<sup>6</sup> cpm/ $\mu$ l) using the conditions described above.

**Expression of Recombinant hVDR in COS-1 Cells.** Two cDNA clones,  $\lambda$ VDR1 and VDR3, were joined at a common restriction site (*Stu* I) and cloned into the expression vector P91023B (18) recreating the full-length coding sequence for hVDR. COS-1 cells were transfected with 12  $\mu$ g of expression plasmid DNA per 100-mm plate using DEAE-dextran (19). After 48 hr, cytosols from 2 $\times$  10<sup>7</sup> cells were prepared by Dounce homogenization in 10 mM Tris-HCl, pH 7.6/0.3 M KCl/5 mM dithiothreitol (designated TK0.3D buffer). Cytosols were diluted to 4 $\times$  10<sup>6</sup> cell equivalents per ml, and 0.1-ml aliquots were incubated with increasing concentrations of 1 $\alpha$ ,25-dihydroxy[26,27-methyl-<sup>3</sup>H]D<sub>3</sub> [1,25(OH)<sub>2</sub><sup>3</sup>H]D<sub>3</sub>] (176 Ci/mmol, Amersham; 1 Ci = 37 GBq) with or without a 100-fold molar excess of unlabeled 1,25(OH)<sub>2</sub>D<sub>3</sub>. After incu-

Abbreviations: VDR, vitamin D<sub>3</sub> receptors; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; hVDR, human VDR; 1,25(OH)<sub>2</sub><sup>3</sup>H]D<sub>3</sub>, 1 $\alpha$ ,25(OH)<sub>2</sub>[26,27-methyl-<sup>3</sup>H]D<sub>3</sub>; mAb, monoclonal antibody.

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<sup>§</sup>The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Intelligenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03258).

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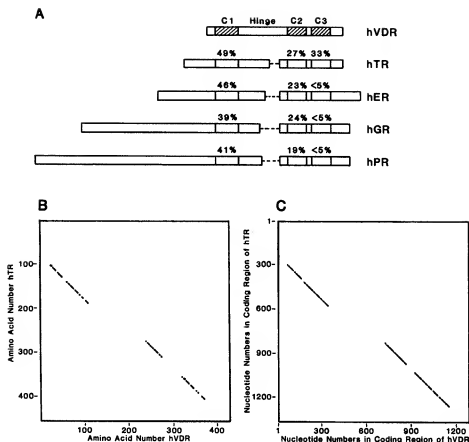


FIG. 3. (A) Schematic comparison between the deduced amino acid sequences of hVDR, human glucocorticoid (hGR), human estrogen (hER), human thyroid hormone (hTR), and human progesterone (hPR) receptors (5–9). Sequences were aligned for maximum similarity about their DNA binding (C1) or C2 domains. Percent similarities indicated are with respect to hVDR sequence. (B and C) Dot matrix plot of the regions of nucleotide- and amino acid-sequence similarity between hVDR and human thyroid hormone receptor.

into the expression vector P91023B in both orientations. This vector uses the adenovirus major late promoter and simian virus 40 origin of replication upstream of the cloned sequences with the mouse dihydrofolate reductase coding region and simian virus 40 polyadenylation signal downstream (18). The final expression constructs, pHVDR1 and pHVDR2, contain 2.1 kb of hVDR sequence in either the correct or inverted orientation with respect to the promoter. These two plasmids were used to transfect COS-1 monkey kidney cells, and the resulting transient pools were analyzed for receptor expression.

A competitive binding assay was used to assess the hormone binding properties of the expressed protein. Cytosolic extracts were incubated with  $1,25(\text{OH})_2\text{D}_3$  with or without a 100-fold molar excess of unlabeled steroid, and receptor-hormone complexes were measured using a hydroxylapatite binding assay (20). The pHVDR1-transfected cells exhibit substantial  $1,25(\text{OH})_2\text{D}_3$ -binding activity, which is saturable at 1 nM. Scatchard analysis (Fig. 4A) indicates the expression of  $\approx 220,000$  copies of hVDR per cell with a  $K_d$  of  $5.2 \times 10^{-11}$  M. The binding affinity of the steroid for the recombinant hVDR is identical to that reported for vitamin D receptors from a number of sources (3, 30), although the level of receptor expression is some 20-fold higher than that found in receptor-rich cell lines such as 3T6 (30) and T47D (31). The results in Fig. 4B illustrate that recombinant hVDR displays selectivity for  $1,25(\text{OH})_2\text{D}_3$  over such metabolites as 25-hydroxyvitamin  $\text{D}_3$ , 24(R),25-dihydroxyvitamin  $\text{D}_3$ , 1 $\alpha$ ,24(R),25-trihydroxyvitamin  $\text{D}_3$ , 1 $\alpha$ -hydroxyvitamin  $\text{D}_3$ , and vitamin  $\text{D}_3$ . The resulting rank order of receptor preference for individual vitamin D metabolites is identical to that of wild-type receptor in all tissues and cells examined to date (31, 32).

Lastly, sedimentation of  $1,25(\text{OH})_2\text{D}_3$ -labeled extracts on 10–30% hypertonic sucrose gradients revealed a receptor

species sedimenting at 3.2S. Preincubation with a mAb to cVDR shifted this peak to 7S (Fig. 4C); both peaks are completely displaceable with excess unlabeled  $1,25(\text{OH})_2\text{D}_3$ . These results are consistent with those found for native hVDR (33).

## DISCUSSION

In this paper we have presented the complete nucleotide sequence of the cDNA for hVDR. Expression of hVDR cDNA in COS-1 cells confirms that the protein encoded by the cDNA represents the authentic receptor on the following criteria. (i) The protein binds  $1,25(\text{OH})_2\text{D}_3$  with the same affinity as does the native receptor. (ii) The expressed protein exhibits the same rank order binding preferences for other vitamin D metabolites. (iii) It sediments on sucrose gradients at 3.2S and displays the characteristic shift to 7S with receptor antibody.

$1,25(\text{OH})_2\text{D}_3$  is thought to act via this receptor to effect changes in gene expression, much like the steroid receptors. However, this has been difficult to confirm because the VDR is very low in abundance and few VDR-regulated genes have been identified. The nucleotide sequence data and the deduced amino acid sequence show that considerable similarity exists among VDR, the steroid hormone receptors, and the thyroid hormone receptor. In particular, a cysteine-, lysine-, and arginine-rich domain is  $\approx 40\%$  conserved between VDR, thyroid receptor, and the steroid receptors. This domain seems capable of forming a  $\text{Zn}^{2+}$ -dependent "finger" and probably harbors both DNA binding and transcriptional activities of the receptors. The presence of this common structural motif in the VDR argues that the mechanism of vitamin D action truly parallels that of the other hormone systems. Furthermore, it has been suggested that VDR and thyroid hormone receptor are closely related biochemically, based

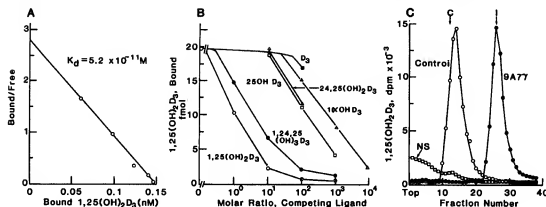


FIG. 4. Expression of hVDR cDNA in transiently transfected COS-1 cells. (A) Saturation analysis of recombinant hVDR in phVDR1-transfected COS-1 cells. The scatchard analysis shows that the expressed receptor has a  $K_d$  of  $5.2 \times 10^{-11}$  M and a  $B_{max}$  of  $2.2 \times 10^5$  sites per cell. (B) Vitamin  $D_3$  metabolite specificity of hVDR. Transfected COS-1 cell cytosol was incubated with  $1,25(OH)_2[P]H_2D_3$  and the indicated molar concentrations of 25-hydroxy-, 24,25-dihydroxy-, 1 $\alpha$ -hydroxy-, 1 $\alpha$ ,24,25-trihydroxy-vitamin  $D_3$ , or vitamin  $D_3$  for 18 hr at  $4^\circ C$  followed by assay using hydroxyapatite (18). (C) Sedimentation analysis of recombinant hVDR. Cytosol was prepared 48 hr after transfection and incubated for 4 hr at  $4^\circ C$  with  $1,25(OH)_2[P]H_2D_3$  (1 nM) with (●) or without (○) 9A7 mAb. A similar incubation was done with  $1,25(OH)_2[P]H_2D_3$ , but this was done with a 100-fold molar excess of  $1,25(OH)_2D_3$  and without the mAb (□). C, cytosol with labeled  $D_3$ ; I, cytosol plus mAb with labeled  $D_3$ ; NS, cytosol with excess unlabeled  $D_3$ .

largely on their strong nuclear binding characteristics (34). The overall sequence similarities presented here support a close evolutionary relationship between these two receptors.

Steroid receptors interact with enhancer-like sequences usually found in the 5'-flanking DNA of regulated genes (35). However, little is known about the sequences involved in the presumed genomic action of the VDR. The availability of cloned receptor sequences will expedite studies of vitamin D-responsive regulatory elements. In addition, expression of truncated receptors (unpublished work) and examination of aberrant receptors found in vitamin D-dependent rickets type II (36) will help to define functional domains of the receptor molecule.

This work was supported by California Biotechnology Inc., by National Institutes of Health grants to J.W.P., M.R.H., and B.W.O., and by March of Dimes Birth Defects Foundation Grant 1-1009 (to J.W.P.). J.W.P. is an established investigator of the American Heart Association.

- Haussler, M. R. & McCain, T. A. (1977) *N. Engl. J. Med.* **297**, 974-978, 1041-1050.
- Pike, J. W. (1985) *Nutr. Rev.* **43**, 161-168.
- Haussler, M. R. (1986) *Annu. Rev. Med.* **6**, 527-562.
- McDonnell, D. P., Mangelsdorf, D. J., Pike, J. W., Haussler, M. R. & O'Malley, B. W. (1987) *Science* **235**, 1214-1217.
- Hollenberg, S. M., Weinberger, C., Ong, W. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfield, M. G. & Evans, R. M. (1985) *Nature (London)* **318**, 635-641.
- Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y. & Shine, J. (1986) *Science* **231**, 1150-1154.
- Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P. & Chambon, P. (1986) *Nature (London)* **320**, 134-139.
- Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, O. J. & Evans, R. M. (1986) *Nature (London)* **324**, 641-646.
- Misrahi, M., Atger, M., d'Auriol, L., Loosfelt, H., Fridlansky, F., Guichon-Mantel, A., Galibert, F. & Milgrom, E. (1987) *Biochem. Biophys. Res. Commun.* **143**, 740-748.
- Huynh, T. V., Young, R. A. & Davis, R. W. (1985) in *DNA Cloning: A Practical Approach*, ed. Glover, D. (IRL, Oxford), Vol. 1, pp. 49-78.
- Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641-646.
- Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237-251.
- Okayama, H. & Berg, P. (1982) *Mol. Cell. Biol.* **2**, 161-170.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl.*

- Acad. Sci. USA* **74**, 5463-5467.
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294-5299.
- Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408-1412.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
- Wong, G. G., Wittek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxemburg, D. P., Jones, S. S., Brown, E. L., Kay, R. M., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R., Hewick, R. M., Wang, E. A. & Clark, S. C. (1985) *Science* **228**, 810-815.
- Deans, R. J., Denis, K. A., Taylor, A. & Wall, R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1292-1296.
- Weckler, W. R. & Norman, A. W. (1979) *Anal. Biochem.* **92**, 314-323.
- Mangelsdorf, D. J., Pike, J. W. & Haussler, M. R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 354-358.
- Pike, J. W., Mangelsdorf, D. J., Allegretto, E. A. & Haussler, M. R. (1987) in *Steroid and Steroid Hormone Action*, eds. Spelsberg, T. C. & Kumar, R. (Nijhoff, Boston), pp. 339-354.
- Pike, J. W. & Sileator, N. M. (1985) *Biochem. Biophys. Res. Commun.* **131**, 378-385.
- Kozak, M. (1985) *Nucleic Acids Res.* **12**, 857-872.
- Weinberger, C., Hollenberg, S. M., Rosenfield, M. G. & Evans, R. M. (1985) *Nature (London)* **318**, 670-672.
- Giguere, V., Hollenberg, S. M., Rosenfield, M. G. & Evans, R. M. (1986) *Cell* **46**, 645-652.
- Miesfeld, R., Rusconi, S., Godowski, P., Maler, B., Okret, S., Wikstrom, A., Gustafsson, J. A. & Yamamoto, K. R. (1986) *Cell* **46**, 389-399.
- Kumar, V., Green, S., Staub, A. & Chambon, P. (1986) *EMBO J.* **5**, 2231-2236.
- Miller, J., McLachlan, A. D. & Klug, A. (1985) *EMBO J.* **4**, 1609-1614.
- Pike, J. W. & Haussler, M. R. (1983) *J. Biol. Chem.* **258**, 8554-8560.
- Freaker, H. C., Marrocci, C., Iwasaki, J. & MacIntyre, I. (1981) *Biochem. Biophys. Res. Commun.* **101**, 1131-1138.
- Putkey, J. A., Weckler, W. R. & Norman, A. W. (1978) *Lipids* **13**, 723-729.
- Mangelsdorf, D. J., Koefler, H. P., Donaldson, C. A., Pike, J. W. & Haussler, M. R. (1984) *J. Cell Biol.* **98**, 391-398.
- Walters, M. R., Hunziker, W. & Norman, A. W. (1981) *Trends Biochem. Sci.* **6**, 268-271.
- Yamamoto, K. R. (1985) *Annu. Rev. Genet.* **19**, 209-252.
- Marx, S. J., Lieberman, U. A., Eil, C., Gambini, G. T., deGrange, D. A. & Balsan, S. (1984) *Recent Prog. Horm. Res.* **40**, 589-611.

# Structural Organization of the Human Vitamin D Receptor Chromosomal Gene and Its Promoter

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The vitamin D receptor (VDR) is known to mediate the pleiotropic biological actions of 1,25-dihydroxyvitamin D<sub>3</sub> through its ability to modulate the expression of target genes. The regulation of this ligand-activated cellular transcription factor is reported to occur at both transcriptional and post-translational levels. To begin to address the molecular basis by which the VDR gene is regulated transcriptionally, we report here an initial characterization of the human VDR gene and its promoter. We isolated several overlapping  $\lambda$ -phage and cosmid clones that cover more than 100 kb of human DNA and contained the entire VDR gene. The gene is comprised of 11 exons that, together with intervening introns, span approximately 75 kb. The non-coding 5'-end of the gene includes exons 1A, 1B, and 1C. Eight additional exons (exons 2-9) encode the structural portion of the VDR gene product. While primer extension and S1 nuclease-mapping studies reveal several common transcriptional start sites, three unique mRNA species are produced as a result of the differential splicing of exons 1B and 1C. The DNA sequence lying upstream of exon 1A is GC rich and does not contain an apparent TATA box. Several potential binding sites for the transcription factor SP1 and other activators are evident. Fusion of DNA fragments containing putative promoter sequences upstream of the luciferase structural gene followed by transient transfection of these plasmids into several mammalian cell lines resulted in significant reporter activity. Due to the size and complexity of the 5'-end of the VDR gene, we examined the activity of a DNA

fragment surrounding exon 1C. An intron fragment 3' of exon 1C conferred retinoic acid responsiveness when fused to a reporter gene plasmid, suggesting a molecular mechanism for the previously observed ability of retinoic acid to induce the VDR. The recovery of the gene for the human VDR will enable further studies on the transcriptional regulation of this gene. (Molecular Endocrinology 11: 1165-1179, 1997)

## INTRODUCTION

The biological actions of 1,25-dihydroxyvitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>] that regulate transcriptional events within the nucleus of target cells are mediated by the vitamin D receptor (VDR)(1-3). This ligand-activated transcription factor belongs to a superfamily of genes that encode receptors for the steroid, thyroid, and vitamin A (retinoic acid and 9 *cis*-retinoic acid) hormones as well as orphan receptor proteins for which ligands have not yet been identified (4-6). Most members of this receptor gene family have undergone considerable scrutiny during the past several years, and significant progress has been made in understanding their structure and function. Although genes that encode proteins involved in mineral metabolism are a principal focus of VDR action and its 1,25-(OH)<sub>2</sub>D<sub>3</sub> ligand *in vivo* (7, 8), the receptor is also capable of influencing genes whose products regulate more fundamental processes of cellular proliferation and differentiation (9, 10). The extensive biological effects now ascribed to 1,25-(OH)<sub>2</sub>D<sub>3</sub> can be attributed, in large part, to the nearly ubiquitous tissue distribution of the VDR as well as to the highly selective regulation of

gene expression by this hormone in specific cell types (1).

Many of the chromosomal genes for the nuclear receptor family members have been cloned and their structural organization determined (11–15). These genes are often more than 50 kb in length and are comprised of multiple protein domain-associated exons separated by exceedingly large introns. An exception to this appears to be the orphan receptors, chicken ovalbumin upstream promoter-transcription factors  $\alpha$  and  $\beta$ , which are encoded by genes that span 4–6 kb of DNA, contain only two introns, and thus may represent ancestral members of this superfamily of genes (16). The promoter regions of several of the receptor genes have been characterized. They often appear to resemble housekeeping genes, and many are embedded in GC-rich islands (11, 13). The absence of a TATA box is a common feature of several of these genes as is the existence of multiple start sites for transcription. In some cases, mRNA transcripts are produced from more than one promoter (17, 18); in others, transcripts are differentially spliced to create unique mRNA species that encode functionally distinct receptor isoforms (19, 20). Thus, nuclear receptor genes can be typified by the general structural organization of the encoding exons as well as through the overall characteristics of many of their promoters.

Transcriptional regulation of nuclear receptor gene expression is clearly evident. While receptors for metabolites of vitamin D and vitamin A, as well as the glucocorticoids are widely expressed (21), receptors for estrogen, progesterone, and androgens display a more restricted tissue-specific expression profile (22). Determinants of restricted expression of these genes in tissues and cells as well as the mechanisms that lead to activation of otherwise silent receptor genes during development or during the differentiation of specific cell types are generally not known. Expressed genes are, however, regulated by a number of non-peptide hormones as well as peptide hormones, growth factors, and cytokines. Transcriptional autoregulation of receptor genes by their respective gene products through *cis* elements localized adjacent to cognate promoters is not uncommon. The  $\beta$ -retinoic acid receptor gene (RAR $\beta$ ), for example, is autoregulated through a  $\beta$ -retinoic acid response element located immediately adjacent to its promoter (23, 24) and accounts for substantial up-regulation of RAR $\beta$  by retinoic acid both *in vivo* and *in vitro* (25, 26).

The VDR is regulated at both transcriptional and posttranslational levels. VDR gene expression is up-regulated in NIH-3T3 mouse fibroblasts through activation of the protein kinase A pathway (27) and down-regulated through activation of the protein kinase C pathway (28). Both of these signal transduction pathways mediate the actions of PTH on target cells (29) and thus may represent homeostatic mechanisms that ultimately control cellular sensitivity to 1,25-(OH) $_2$ D $_3$ . Other growth factors and cytokines are also known to regulate VDR gene expression, possibly through the

above pathways. Estrogens (30), thyroid hormone (31), glucocorticoids (32), and retinoic acid (33, 34) are likewise able to alter VDR mRNA levels in what appear to be tissue-specific patterns of expression. Interestingly, both cell cycle (35) and the differentiation state of cells in culture (36) influence the extent of VDR mRNA expression. The level of the VDR under these circumstances may play a regulatory role in the control of cellular proliferation and differentiation by the vitamin D hormone in a wide variety of cell types. Finally, homologous up-regulation of VDR mRNA by 1,25-(OH) $_2$ D $_3$  has also been demonstrated both *in vitro* and *in vivo*, again in a very tissue-specific fashion (37–39). Whether this regulatory action occurs directly as a result of VDR interaction with a *cis*-element(s) at the 5'-end of the VDR gene, analogous to the interaction of RARs with the RAR $\beta$  gene promoter, or indirectly as a result of the induction or activation of other transcription factors remains to be demonstrated.

To gain a better understanding of the molecular mechanisms by which the VDR is regulated at the transcriptional level, we cloned the chromosomal gene for the human VDR. We report here on structural characterization and preliminary functional activity of its promoter. The single gene for human VDR spans more than 75 kb of genomic DNA and contains 11 exons. Three exons make up the 5'-noncoding region, and the eight additional exons encode the structural component of the VDR. The promoter is characterized by the lack of a TATA box initiator, its GC-rich nature, and the presence of putative binding sites for SP1 and a variety of transcription factors. It directs the transcription of at least three VDR mRNA transcripts in kidney that appear to arise from the differential splicing of 5'-noncoding exons. The promoter also directs the transcription of a chimeric gene when fused upstream of an expression plasmid containing the luciferase structural gene. The recovery of the human VDR gene and these initial studies provide the basis for further detailed examination of the transcriptional regulation of the VDR gene. This effort is particularly warranted in view of the central role of the VDR in 1,25-(OH) $_2$ D $_3$  action and the potential therapeutic role of 1,25-(OH) $_2$ D $_3$  and 1,25-(OH) $_2$ D $_3$  analogs in blocking cellular proliferation and promoting the differentiation of tumor cells (40, 41).

## RESULTS

### The Structure and Sequence of hVDR mRNAs

We established first the authenticity of the 5'-end of the hVDR mRNA sequence reported initially by Baker *et al.* (42) using the 5'-RACE (rapid amplification of cDNA ends) PCR technique. Human kidney RNA was employed as template to prepare first-strand cDNA, and the latter was amplified using an anchored oligonucleotide (primer A) as well as a downstream primer (primer B2) corresponding to hVDR cDNA sequence as outlined in Fig. 1A. Primary amplification was fol-



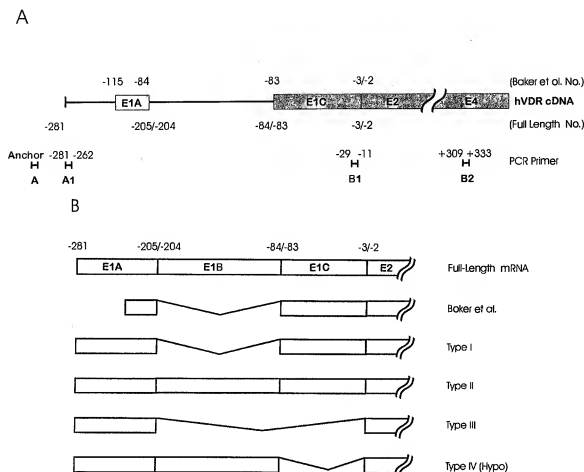


Fig. 1. Analysis of hVDR mRNA Transcripts Using 5'-RACE

A. Location of oligonucleotide primers used for DNA amplification. The shaded rectangle illustrates portions of the hVDR transcript originally reported by Baker *et al.* (42), whereas the single line indicates additional portions of the hVDR mRNA transcript revealed through 5'-RACE. The numbering above the diagram corresponds to that of Baker *et al.* (42) whereas the numbering below corresponds to the largest hVDR mRNA transcript that was identified. The location of DNA primers used to characterize the hVDR mRNA transcripts by 5'-RACE are indicated relative to the largest unspliced hVDR mRNA transcript identified. The anchor primer (corresponding to vector sequence) was obtained from Life Sciences. B. The three hVDR mRNA transcripts identified through the 5'-RACE technique are illustrated (type I, II, and III) relative to the transcript identified by Baker *et al.* (42). Type I and type II transcripts contain deletions that correspond to proposed exon 1B (type I) and exons 1B and 1C (type II), respectively. The type III transcript contains all the proposed exons: 1A, 1B, and 1C. Exon 2 (43) is common to all transcripts. The type IV transcript is hypothetical. The illustration is numbered relative to the full-length transcript (type II).

lowed by a secondary amplification as described in *Materials and Methods* using primers A and B1. The cloning and DNA sequence analysis of the resulting products revealed two individual transcripts. As observed in Table 1, the sequence of the first (type 1) was identical to that reported by Baker *et al.* (42) with the exception that the sequence of the DNA product extended some 45 bp upstream. This suggests that the original cDNA sequence reported by Baker *et al.* (42) was incomplete at the 5'-end by 45 bases and that the type 1 PCR product might represent the authentic 5'-end of the hVDR mRNA. The second transcript (type 2) was identical to the type 1 transcript (see Table 1) with the exception that a 121-bp insert was discov-

ered lying between nucleotides -84 and -83 relative to the translation start site reported by Baker and colleagues (42). An additional amplification of kidney RNA was then carried out using primer A1 complementary to the authentic 5'-end of the mRNA established above and primer B2 as illustrated in Fig. 1A. Cloning and sequence analysis of the products of this reaction revealed the previous two mRNAs as well as a third (type 3). As documented in Table 1, type 3 was similar to type 1 with the exception that nts -83 to -3 relative to the sequence of Baker *et al.* (42) were deleted. The failure of primer B1 to hybridize to type 3 transcripts clearly prevented detection of type 3 in the original primary amplification described above. The

**Table 1. Nucleotide Sequences of hVDR in RNA Transcripts Detected by 5'-RACE**

Baker <i>et al</i>	5'-GGAAAGCTTT GTCCACCGC CGGCCGACC AGAAGCTTT GGGTCTGAAG	+50
	TGTCTGTGAG ACCTCACAGA AGAGCACCCC TGGGCTCAC TTACCTGCC	+100
	CCTGCTCCTT CAGGGATG-3'	+150
Type I	5'-CTGCTTGTC AAGGCGGCA GCGAGCCGT GTGCGCGGG AGCGCGAAG	+50
	AGCTTGTC ACGCGCGCC GGACCAAGAG CTTTGGGTC TGAAGTGTCT	+100
	GTGAGACCTC ACAAGAGAG ACCCTTGGG TCCACTTACC TGCCCCCTGC	+150
	TCCTTCAGGG ATG-3'	+200
Type II	5'-CTGCTTGTC AAGGCGGCA GCGAGCCGT GTGCGCGGG AGCGCGAAG	+50
	AGCTTGTC ACGCGCGCC GGACCAAGAG CTTGAACTTA GCCCAGCTGG	+100
	ACGAGAAAT GGACTCTAGC CTCTCTGAT AGCTCATGC CAGGCCCTGT	+150
	GCTCATGTCT TTGCTTGGCT CCTCTAATCC TCATAGCTTC TCTTTGGGAA	+200
	CCCTTTGGGT CTGAAGTGTG TGTGAGACCT CACAGAAGAG CACCCCTGGG	+250
	CTCCACTTAC CTGCCCCCTG CTCTCTCAGG GATG-3'	+300
Type III	5'-CTGCTTGTC AAGGCGGCA GCGAGCCGT GTGCGCGGG AGCGCGAAG	+50
	AGCTTGTC ACGCGCGCC GGACCAAGGA TG-3'	+100

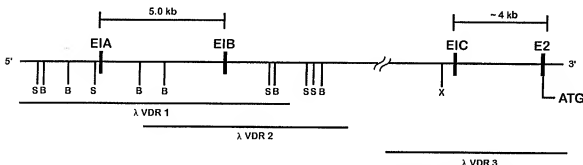
The preexisting sequences documented by Baker *et al.* (42) are indicated in **bold**. Accession numbers for these sequences are AB002157-AB002168.

organization of the three transcripts are illustrated in Fig. 1B relative to the sequence of Baker *et al.* (42). The nature of these transcripts, one of which contains an insertion and the other a deletion, suggests that the 5'-end of the hVDR gene contains four exons, three of which are exclusively noncoding exons, and that at least two of these exons may undergo unique splicing events in the kidney. Based upon these data, a fourth unique transcript containing a deletion of exon 1C is possible.

#### λ-Phage Genomic Clones

Having characterized the 5'-end of the hVDR mRNA, we used the 5'-RACE-derived DNA fragments to screen a λ-phage genomic library and recovered several positive

clones. Three clones were mapped using the restriction enzymes *Bam*HI, *Eco*RI, and *Sac*I, and relevant probe-fragments were sequenced. As observed in Fig. 2, λ-clones 1 and 2 represent overlapping clones that contain exons 1A and 1B, which are in turn separated by an intron of approximately 5 kb. λ-Clone 3 did not overlap λ-clone 2 but contained exon 1C and exon 2. Exon 2 contained the start site of translation beginning 3 bp downstream of the 5'-end of the exon. The intron located between exons 1C and 2 was determined to be approximately 4 kb, but the lack of contiguity between λ-clones 2 and 3 prevented determination of the size of the intron located between 1B and 1C. The presence of these four exons is entirely consistent with the data obtained from analysis of the hVDR mRNA. We designated the first three exons 1A, 1B, and 1C to be consistent with a



**Fig. 2. Organizational Map of Exons 1A, 1B, 1C, and 2 Comprising the 5'-End of the hVDR Chromosomal Gene**

A λ-phage genomic library was screened using 5'-RACE products to obtain three genomic fragments containing the hVDR gene. Each clone was restriction mapped and relevant portions sequenced. Restriction mapping and sequence analysis revealed that two overlapping clones (λ-VDR1 and λ-VDR2) contained sequence corresponding to proposed exons 1A and 1B (type II) separated by 5 kb (intron A). A downstream noncontiguous clone (λ-VDR3) contained exons 1C and exon 2 (type II) separated by 4 kb (intron B). The distances between exons 1A and 1B and between exons 1C and 2 were determined by restriction mapping followed by Southern blot analysis. S, *Sac*I; X, *Xho*I; B, *Bam*HI. ATG represents the first translation codon.

preliminary description of the organization of this gene (43).

### Cosmid Genomic Clones

In view of the lack of contiguity between  $\lambda$ -clones 2 and 3, and to recover the remainder of the hVDR gene, we screened a human liver genomic library prepared in the cosmid vector pCV109 (44) sequentially with several nick-translated hVDR cDNA probes. Screening resulted in the recovery of four unique individual human DNA cosmid clones designated phVDRG1, 3, 4, and 11. As illustrated in Fig. 3A, EcoRI, KpnI, XhoI, and SalI restriction endonucleases were used initially to map each of the respective clones and revealed that they represent overlapping fragments of 40 to 50 kb which together span approximately 93 kb of contiguous genomic DNA.

Three unique hVDR cDNA probes (see *Materials and Methods*) were used to determine the extreme 5'- and 3'-boundaries of the VDR gene within this locus and to determine the relative orientation of individual DNA restriction fragments located within these four clones with respect to the hVDR mRNA. Southern blot hybridization analysis with these respective VDR cDNA probes revealed that clones G1, G3, and G11 likely contained exons comprising the entire VDR chromosomal gene. Subsequent detailed hybridization analysis of G3 and G11 DNA digested with EcoRI, KpnI, or EcoRI and KpnI utilizing hVDR cDNA probe 3 (nt \*10 to \*2012 which includes the entire normal hVDR open reading frame) revealed the presence of eight hybridizable DNA fragments spanning a total of 55 kb (Fig. 3B, left). The locations of these fragments (identified as B4.2, C2.2, D4.4, E8.0, F5.5, G5.6, H2.7, and I7.0) in relationship to the genomic cosmid clones are depicted in Fig. 3A. Importantly, each of these DNA restriction fragments was unequivocally identified in human genomic DNA after high-stringency Southern blot hybridization analysis with a combination of the cDNA probes indicated above. Six of these fragments (C-H) are detected after analysis of EcoRI- and KpnI-digested human DNA with probe 3 (Fig. 3B, right).

The position of exon 1A of 77 bases in genomic clone phVDRG1 was verified through hybridization screening with an oligonucleotide corresponding to a portion of exon 1A. DNA fragment A was sequenced to identify the first exon located as illustrated in Fig. 3A. Exons 1B (121 bases), 1C (81 bases), and 2 (148 bases) located in this clone were mapped relative to exon 1A, further defining the approximately 20 kb size of the intron located between exons 1B and 1C (see figure). DNA fragments B and C (see Fig. 3A) were cloned and sequenced to identify the intron/exon boundaries of exons 1C and 2. Exon 1B was positioned relative to exon 1A in  $\lambda$ -clone 1.

Additional DNA fragments identified through hybridization analysis were isolated, subcloned, and either partially or completely sequenced to determine the precise location of downstream VDR exons. As observed in Fig. 3C, the gene is split into seven additional

exons (exons 3 through 9) of 131, 185, 121, 172, 152, 117, and 3466 bp, which, together with the upstream exons, form the authentic full-length hVDR gene. The gene itself, however, spans some 75 kb of DNA, the majority consisting of introns whose sizes, boundaries, and locations within the mature hVDR mRNA are documented in Table 2. A comparison of the sequences of the intron/exon boundaries indicates that each conforms to a canonical splice consensus sequence typical of most eukaryotic genes.

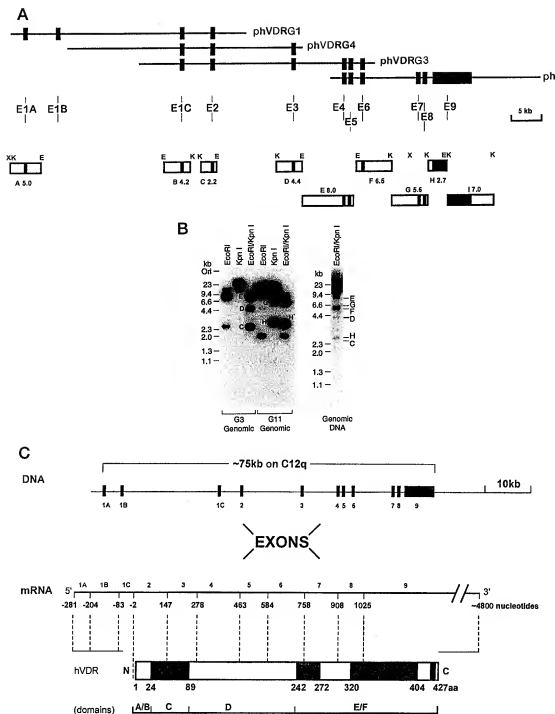
The relative organization of the exons and their locations with respect to domains located in the hVDR protein (45) is depicted in Fig. 3C. Exon 2 contains 2 bp of noncoding sequence, the translation initiation codon, and the N terminal of the two dissimilar DNA-binding Zn<sup>2+</sup> finger modules. Exon 3 lies downstream of a 15-kb intron and encodes the second DNA-binding zinc module. Exons 4-6 are clustered together; exons 4 and 5 encode a region that serves a hypothetical hinge function between DNA- and steroid-binding portions of the receptor protein, while exon 6 encodes the remainder of the hinge and the first portion of the steroid-binding domain. Finally, clustered exons 7-9 encode the C-terminal half of the VDR, with exon 9 containing the final 85 amino acids as well as the remaining approximately 3200 bp that constitute the large 3'-noncoding sequence.

### An Exon 2 Translation Site Polymorphism

Exon sequences within the VDR gene derived here were compared with those of the human VDR cDNA recovered from the T47D breast cancer cell line and reported by Baker et al. (42). Exon sequences corresponded to those found in the hVDR cDNA with one exception, a T to C transition that eliminated the most 5'-ATG codon within the T47D VDR mRNA (see Table 2). The likely result of this mutation, which creates a potential polymorphic FokI site within exon 2, is the utilization of a second in-frame translation codon beginning 10 nucleotides downstream that ultimately encodes a potentially foreshortened receptor protein of 424 amino acids. Evaluation of additional human DNA samples via DNA amplification techniques revealed that this feature of the cloned gene was not unique to the genomic library from which the hVDR gene was recovered (data not shown). More importantly, perhaps, the existence of an hVDR mRNA of the latter type has been demonstrated recently by Saijo et al. (46). Despite these observations, however, it will be necessary to confirm that two proteins that differ in molecular mass by only three amino acids are in fact translated in human tissues or cells.

### The hVDR Promoter

We sequenced a DNA fragment extending 5' of the start site of transcription containing the putative promoter for hVDR (Fig. 4). While this promoter lacks consensus TATA or CAAT boxes, the region is GC rich with five binding motifs for the transcription factor SP1 lying between



**Fig. 3. Organizational Map of the Complete hVDR Chromosomal Gene**

A, Organizational map of the complete hVDR chromosomal gene. A human liver cosmid library was screened with hVDR cDNA probes to obtain four clones (phVDRG1, phVDRG4, phVDRG3, and phVDRG11) that spanned the entire hVDR gene. Restriction mapping and Southern blot analysis revealed the overlapping nature of the clones as well as the relative position of each exon within the clone as indicated. Relevant restriction fragments (A5.0, B4.2, C2.2, D4.4, E8.0, F5.5, G5.6, H2.7, and I7.0) indicated as rectangles were subcloned and subjected to high resolution restriction mapping and sequencing. X, *Xho*I; K, *Kpn*I; E, *Eco*RI. The positions of the hVDR exons, which are numbered E1A, E1B, E1C, E2-E9, are indicated by the black bars. B, Southern blot analysis of phVDRG3 and phVDRG11 DNA and human genomic DNA. Overlapping phVDRG clones 3 and 11 were subjected to restriction digestion with *Eco*RI, *Kpn*I, or both enzymes as indicated (left). Human genomic DNA was subjected to simultaneous

**Table 2.** Features of the hVDR Gene

No.	Exons		Exon/Intron Sequence			
	Size (bp)	Position	5'-Exon Boundary	Intron (Size, kb)	3'-Exon Boundary	
1A	77	-281/-205	CGGACCAggtgcgaaccc	... A (4.95)	... tatccttaagGCTCTGAACCTAGC	
1B	121	-204/-84	TCCTTGGGgtaagtacag	... B (-20)	... ttcatttcagAAGCCTTTGGGCTCG	
1C	81	-83/-3	TCCTTCAggtaggtgttt	... C (-4)	... gttccttacagG	ATG GAG GCA ATG Met Glu Ala Met
2	148	-2/146	TTC AGgtgagcccttc Phe Ar 49	... D (13.2)	... ctctcggcagG	CGA AGC ATG Arg Ser Met
3	131	147/277	GAG Tgtgagtgctcc Glu P 93	... E (8.4)	... caccctcacagTC	ATT CTG ACA he lle Leu Thr
4	185	278/462	TTC CGgttatgtctctgc Phe Arg 154	... F (0.252)	... tcccttccagCCT	CCA GTT CGT Pro Pro Val Arg
5	121	463/583	TCA Ggtaagcagga Ser A 195	... G (1.32)	... tctctccagAC	ATG ATG GAC sp Met Met Asp
6	172	584/755	TTC AGgtaagaaacc Phe Ar 252	... H (9.2)	... tcccttcagA	GAC CTC ACC g Asp Leu Thr
7	152	756/907	AAA Ggtatgcctag Lys A 303	... I (0.205)	... tctctcacagCC	GGG CAC AGC la Gly His Ser
8	117	908/1024	CCA Ggtatggggcc Pro A 342	... J (-1.4)	... gtgccacagAT	CGT CCT GGG sp Arg Pro Gly
9	3466	1025/3' END				342

The size and position of each of the 11 exons relative to the hVDR mRNA type II transcript are documented. The intron/exon boundaries as well as the approximate size of the introns are indicated. The canonical splice site consensus sequences are indicated in *bold*. The polymorphism associated with the two start sites of translation are *underlined* and shown in *bold*.

nucleotide (nt) -72 and -34 relative to the transcription start site (47). Potential binding sites for other transcription factors are also evident (48). In addition, five AG-GTCA-like sequences, which represent potential nuclear receptor-binding element half-sites (49), are located between nt -1394 and -949. The functional relevance of these, as well as of additional interesting sequences, will need to be determined.

#### Activity of the 5'-Flanking Region of the hVDR Promoter

To evaluate the transcriptional capacity of the hVDR promoter, we cloned a series of 5'-deletion frag-

ments of the gene into a luciferase reporter gene and transfected them individually into two mammalian cell lines. We tested constructs beginning at -1.935, -1.479, -1.221, -0.586, -0.464, -0.103, -0.034 kbp relative to the start site of transcription. The downstream boundary of each construct was located at the 3'-boundary of exon 1A at +71 relative to the start site of transcription [-89 relative to the start site of translation reported by Baker *et al.* (42)]. As observed in Fig. 5A, pVDE1-1.93 was capable of directing significant luciferase expression when introduced by transient transfection into HeLa cells. Each deletion construct likewise exhibited

digestion with *KpnI* and *EcoRI* (right). Southern blot analysis was carried out as described in *Materials and Methods* and hybridized to the hVDR3 probe. Bands D-H identified both in genomic DNA and within the clones represent the DNA fragments schematically illustrated in A. Band C was weakly evident on the autoradiogram but not reproducible in the figure. C, Structural organization of the human chromosomal vitamin D receptor gene. The structural organization of the human VDR gene locus (DNA) comprising 11 exons (1A, 1B, 1C, 2 through 9) spanning approximately 75 kb of DNA is depicted. A 10 kb scale is indicated to the right. The location of exons relative to the mRNA transcript of ~4800 nucleotides (mRNA) and the encoded VDR protein of 427 amino acids (hVDR) is illustrated. With regard to the hVDR mRNA, negative numbers indicate 5'-noncoding nucleotides, and positive numbers indicate protein encoding nucleotides beginning with +1 indicated by Baker *et al.* (42) as well as 3'-untranslated sequences. Numbers below the hVDR protein indicate the amino acid residue boundaries of shaded homology domains. Regions of functionality are designated A/B, C, D, and E/F as described in Ref. 76.

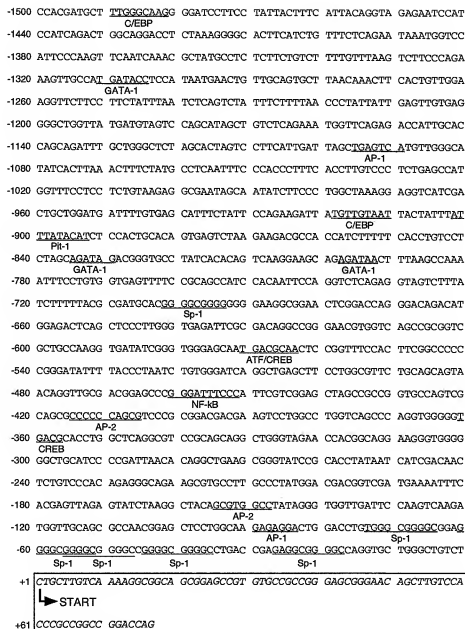
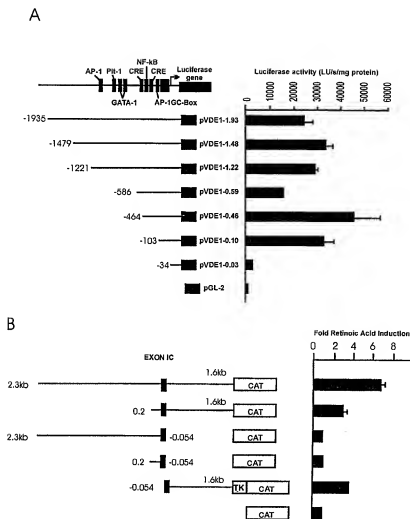


Fig. 4. Sequence Determination of the hVDR Gene Promoter

The nucleotide sequence of the hVDR gene promoter is documented. The transcription start site is indicated as +1 beginning within the boxed sequence. The locations of potential binding sites for specific transcriptional regulators are underlined and indicated. Transcription factors indicated include SP-1, AP-1, AP-2, NFkB, GATA-1, Pit-1, and C/EBP. Potential binding sites for nuclear receptors are also present.

substantial transcriptional activity, although this activity varied and did not exhibit an obvious pattern. Constructs containing proximal elements of the promoter (-464 and -103 relative to the start site) displayed the most activity, suggesting the possibility that elements upstream of -0.464 kb may confer a negative regulatory function. 5'-Deletion of the hVDR promoter from -103 bp to -34 bp that re-

moved four of the five GC boxes near the initiator resulted in a 10-fold drop in activity. The latter result substantiates the hypothesis that these elements play an important role in the activity of the hVDR promoter and provide the basis for future studies. Preliminary studies in Hela cells suggest that the 5'-flanking region of the gene is not responsive to 1,25-(OH)<sub>2</sub>D<sub>3</sub> (data not shown).



**Fig. 5. Functional Analysis of the Human VDR Gene Promoter**

A, hVDR promoter constructs pVDR-1.93, pVDR-1.46, pVDR-1.22, pVDR-0.589, pVDR-0.46, pVDR-0.10, pVDR-0.03, and the promoterless control plasmid (pGL-2) were individually cotransfected together with the normalization vector pCMV- $\beta$ -galactosidase as indicated in *Materials and Methods*. The activities of these constructs in HeLa cells were determined after 64 h and normalized to the activity of  $\beta$ -galactosidase. Luciferase activity is expressed as light units per second per mg of protein. Data are means  $\pm$  SEM of four independent experiments. B, DNA fragments surrounding exon 2 as indicated in the figure were fused to the pBL CAT2 or pBLCAT3 chloramphenicol acetyltransferase expression vectors and transfected into rat ROS 17/2.8 cells as described in *Materials and Methods*. Transfected cells were treated with either vehicle or retinoic acid ( $10^{-6}$  M) for 48 h after which the cells were harvested and extracts (100  $\mu$ g protein) assayed for CAT activity. Each assessment represents the mean  $\pm$  SEM of a triplicate determination. The data are representative of three separate experiments and are expressed as the activity of the construct in the presence of retinoic acid compared with the activity of the construct in the ligand's absence (fold induction).

### A Retinoid-Responsive Region Lies Downstream of Exon 2

In view of the complexity of the hVDR gene, we examined several DNA fragments surrounding exon 1C for their capacity to direct transcriptional activity or to be regulated by hormones such as 1,25-(OH)<sub>2</sub>D<sub>3</sub> or retinoic acid. All clones examined exhibited transcriptional activity when cloned into the promoterless chloramphenicol acetyltransferase (CAT) vector pBLCAT3 (data not shown). As observed in Fig. 5B,

however, several constructs, each containing intron sequence downstream of exon 2, were responsive to treatment with retinoic acid ( $10^{-6}$  M). The constructs were uniformly unresponsive to  $1,25\text{-(OH)}_2\text{D}_3$  in this cell line (data not shown). The ability of this fragment of DNA to transfer retinoic acid response to the viral thymidine kinase promoter provides further support for the possibility that the previously identified regulation of VDR expression by retinoic acid (33, 34) is direct and mediated via a *cis*-element located a sig-

nificant distance downstream of the authentic promoter. Further work will be required to define the exact location of this *cis*-element.

## DISCUSSION

We report here the structural organization of the human VDR gene derived from several  $\lambda$ -clones and four overlapping cosmid clones that span the entire gene locus. The human gene comprises 11 exons that together with the associated introns cover approximately 75 kb of DNA. Three exons (exons 1A, 1B, and 1C) make up the 5'-noncoding leader sequence of the largest of the hVDR mRNA species (type 2). An additional eight exons (exons 2-9) encode the structural portion of the gene product. This numbering system allows retention of the numbering system assigned in an earlier preliminary analysis of the gene (43) as well as that found in a body of literature related to the existence of hVDR gene polymorphisms that has evolved recently (see below). The multiple exonic structure and general organization of the hVDR gene are comparable to that of many of the other steroid receptor genes that have been characterized including the thyroid receptor (18), progesterone receptor (11), estrogen receptor (12), androgen receptor (14), and glucocorticoid receptor (13, 17). The promoter for this gene is TATA-less and GC rich.

The organization of the hVDR gene indicates that separate exons encode each of the zinc finger modules and that the 3'-boundaries of each of these exons appear to be generally conserved within the nuclear receptor family of genes. One exception may be chicken ovalbumin upstream promoter-transcription factor  $\alpha$  and  $\beta$ , for which both finger modules appear to be encoded by a single exon (16). Although the two zinc modules within these proteins appear highly related structurally, they are not equivalent topologically (50). Furthermore, the function of each module is substantially different; the first zinc module determines the specificity of DNA binding whereas the second is more intimately involved in the protein-protein contacts that stabilize the association through dimerization (51). Although it is possible that the two exons encoding these modules evolved from a common ancestral gene through duplication and subsequently diverged under a different set of selective pressures, it is also possible that they evolved independently. Three exons encode the hVDR hinge, whereas only two encode this flexible region within the sex steroid receptor genes. It is noteworthy that the VDR appears to contain an extended hinge region relative to many of the other members of this gene family (4). The function, if any, of these additional hVDR residues that are encoded by insertion of exon 5 is unknown, although this region appears to be the least conserved among VDRs from different species. Finally, exon 2 of the hVDR is restricted to 21 amino acids upstream of the first zinc

module, placing the hVDR DNA-binding domain near the amino-terminal end of the protein. This is unlike several of the other family members where one or more relatively large exons lie upstream, relegating the DNA-binding domain to a more central location within the protein (4). The absence of this extended region within the hVDR, whose presence in the larger receptors is associated with an important activation domain(s) (52-54), implies a more complete reliance of the hVDR on the carboxy-terminal activation function (AF2) or on an, as yet, undescribed activation region.

Amplification of the 5'-end of the hVDR mRNA revealed the existence of three separate transcripts in human kidney RNA. Because each contains the identical start site and first exons, the transcripts are likely derived from alternative splicing of two of the 5'-noncoding exons, 1B and 1C, one of which eliminates exon 1B and the other which eliminates both exons 1B and 1C. It is hypothetically possible that a fourth transcript that does not contain exon 1C also exists, perhaps in tissues other than kidney. Alternative splicing of the RAR, retinoid X receptor, thyroid hormone receptor, and other receptors is a common feature of this gene family of proteins. Unlike that which was observed here, some splicing events lead to the productions of different proteins with unique functions (19, 20). The relative abundance of each of the three hVDR transcripts within the kidney is unknown; indeed, the similarity in their size prevented their earlier detection by Northern blot analysis. The existence and relative abundance of these hVDR transcripts in other tissues as well as a determination of their possible individual functions remain for future studies.

The promoter region of the hVDR lies in a GC-rich island and does not contain a TATA box. In that respect, the hVDR gene is like certain other steroid receptor gene promoters. Our analysis of this promoter indicates a substantial capacity to direct transcription of a chimeric reporter function. The most proximal region (-103 to -34) imparts more than 80% of the activity of the promoter in HeLa cells and appears to contain strong positive elements. A possible negative contribution is evident between -586 and -464. The hVDR promoter contains an array of putative binding sites for transcription factors that mediate the activities of multiple pathways that serve to transduce a variety of extracellular signals. For example, it is known that the VDR is regulated by both PKA and PKC pathways (27-29, 55, 56) that are in turn known to converge on several specific transcription factors. These and other studies support the possibility that binding sites upstream of the hVDR promoter may play modulatory roles in its regulation, although future functional studies will be necessary to confirm this hypothesis. At the very least, the presence of five GC boxes immediately upstream of the start site of transcription suggests a fundamental role for the transcription factor SP1 in the activation of this gene (47). SP1 is known to interact both with a number of cellular and viral promoters as well as with other transcription



factors such as NF $\kappa$ B (57, 58). Interestingly, the hVDR gene is induced with retinoic acid at a site that lies downstream of exon 1C. While a potential binding site for one or more of the retinoid receptors has not been definitively localized, it is likely that this site mediates the recognized ability of RA to induce the transcription of VDR (33, 34). The existence and, more importantly, the location of this site, together with the size of the introns lying between exons 1A, 1B, and 1C, suggest that it may prove difficult technically to identify additional regulatory sequences. The inability to detect vitamin D inducibility of the VDR gene at the promoter level currently suggests that the autoregulatory actions of vitamin D are indirect. For example, a recent study suggests that 1,25-(OH) $_2$ D $_3$  can stimulate the expression of *c-fos* in osteoblastic cells that might in turn stimulate hVDR gene expression (59). Based upon the noted complexity at the 5'-end of the VDR gene, however, it is possible that 1,25-(OH) $_2$ D $_3$ -responsive portions of the gene have yet to be defined.

During the analysis of the hVDR gene, we identified the existence of a polymorphism associated with exon 2. This polymorphism leads to synthesis of two different hVDR mRNAs which we detected utilizing single-strand conformational analysis (46). One corresponds to the mRNA sequence of Baker *et al.* (42) wherein the start site of translation is situated at codon 1 and the second is associated with a T to C conversion in this codon that results in potential translation beginning at an in-frame ATG located downstream at codon 4. Translation of such an mRNA would result in the production of an abbreviated protein of only 424 amino acids. Two studies have recently reported the distribution of these alleles in the human population (60, 61); the ATG to ACG allele, which can be detected by the presence of a *FokI* site (60), represents the more common form. Interestingly, the frequency of this allele correlates with an increase in bone density in two different human female populations (60, 61). This observation suggests that the VDR products of these two alleles may exhibit unique activities. Validation of this hypothesis, however, will require additional population studies as well as the demonstration that the two VDR alleles are indeed expressed in human tissues and that they exhibit quantitative and/or qualitative differences in their activity on bone or other tissues. As a first step, Arai *et al.* (61) have shown that both proteins can be produced through recombinant means in transfected COS cells. More importantly, the capacity of these two gene products to direct transcription of a vitamin D-sensitive chimeric gene in cotransfected cells is quantitatively different. The smaller protein, whose corresponding allele appears to correlate with increased bone density, exhibited greater transcriptional activity. Additional studies will be necessary to confirm these functional studies as well as the correlation between bone mineral density and VDR alleles. Interestingly, some plasticity is also apparent in these two codons in other species; while the mouse VDR mRNA contains

both ATGs (62), the rat VDR mRNA contains only the most 5'-ATG (63). Perhaps unique activities are also associated with these mRNA products.

Additional polymorphisms have been identified within the hVDR gene in the intron between exons 7 and 8 (*BsmI* and *ApaI*) and within the 3'-noncoding sequences lying in exon 9 (*TaqI*) (64) (see Table 3). The presence of these restriction fragment length polymorphisms has been reported to be associated with population bone mineral density (65) and more recently with prostate cancer (66). In the former case, this finding has not been widely reproduced, suggestive of relatively weak linkage, and remains highly controversial (67). In contrast to the *FokI* polymorphism located in exon 2, it is unclear how the *BsmI* and/or *TaqI* polymorphisms located in non-coding portions of the hVDR gene might influence hVDR function. It is possible that they are linked in some way either to the potentially functional exon 2 start site polymorphism, to as yet unidentified allelic differences located within the hVDR gene promoter, or to unrelated genes that impact bone mineralization directly. Both the validity of the proposed associations between hVDR polymorphisms and disease and, if proven true, the mechanism(s) by which they impart disease remain to be established.

In conclusion, we report on the structural organization of the hVDR chromosomal gene. The availability of this gene locus as well as identification and cloning of its promoter will enable future studies aimed at identifying molecular determinants of the VDR's expression. A wealth of studies that describe the regulation of expression of the VDR provide the backdrop and rationale for these impending studies.

## MATERIALS AND METHODS

### Messenger RNA Analysis

The sequence of the 5'-end of the human VDR mRNA was determined utilizing the 5'-RACE system obtained from Life Technologies, Inc. (Gaithersburg, MD) (68). Total RNA was isolated from human kidney (69, 70) and used to synthesize first-strand cDNA utilizing random hexamers and oligo d(T) primers and Superscript RT (Life Technologies, Inc). Two sequential PCR amplification reactions were employed to identify the 5'-ends of the hVDR mRNAs and to generate DNA fragments useful for initial sequencing. As illustrated in Fig. 1A, the first was performed using common anchored primer A (5'-CTGGTTCGGGCCACCTCTGAAGGTTCAGGAATCGATAG-3') and primer B2 (5'-CTTCGGCTTGAGGATCATCTCCGGC-3'), which corresponds to nt +309 to +333 relative to the translation start site of the human VDR cDNA of Baker *et al.* (42). The second was performed with a portion of the reaction products from the first amplification and used primer A and primer B1 (5'-GCAGGGGGCAGGTAAAGTG-3'), which corresponds to nt -29 to -11 relative to the start site of translation reported by Baker *et al.* (42). Sequence determination of the PCR products of these sequential reactions established the 5'-end of the hVDR mRNA transcript, enabling a subsequent amplification of kidney RNA using primer A1, which corresponds to the first 22 bases of the

authentic 5'-end of the transcript and primer B2 (see Fig. 1A). All amplified DNA products were subcloned into pBluescript II SK (Stratagene, San Diego, CA) and sequenced using the SequiTherm Long-Read Cycle Sequencing Kit-LC (Epicentre Technologies, Madison, WI). All DNA sequencing was performed using the fluorescence-based LI-COR model 4000L sequencer.

#### Recovery and Analysis of hVDR Genomic Clones

A human EMBL 3 genomic library was screened with <sup>32</sup>P-labeled PCR probes obtained after 5'-RACE of human kidney mRNA using high-stringency hybridization conditions. Nylon membrane replicas of approximately 1.5 × 10<sup>6</sup> plaques were incubated for 2 to 3 h in 50% formamide, 5× Denhardt's solution, 0.75 M NaCl, 0.05 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 3 mM EDTA, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA. Hybridization was performed overnight at 42°C with 5 × 10<sup>6</sup> cpm/ml labeled probe. Membranes were washed for 1 h at 30°C in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0, and 0.05% SDS and then twice for 1 h at 68°C in 0.15 M sodium citrate, pH 7.0, and 0.1% SDS. After exposure of the membranes to Kodak X-Mat film, positive plaques were isolated, rescreened twice, and purified, and then the inserts were isolated and subcloned into pBluescript II SK. DNA inserts were verified by Southern blot analysis, restriction enzyme-mapped by routine methods, and portions were then sequenced using the strategy outlined below.

A human liver genomic library prepared in the cosmid vector pCV109 (44) was screened via colony hybridization techniques (71) using nick-translated hVDR cDNA probes. hVDR probe 1 extended from -115 to +145 nt relative to the sequence of Baker et al. (42); hVDR probe 2 contained nucleotides +10 through +576 relative to the sequence of Baker et al. (42); and hVDR probe 3 contained nucleotides +10 through +2012 nt relative to the sequence of Baker et al. (42). The latter probe represents the entire open reading frame of the normal hVDR cDNA together with 731 nucleotides of noncoding 3'-sequence. Nylon filter-immobilized DNA was prehybridized at 68°C overnight in 6× NaCl-sodium citrate (SSC) (1× = 0.15 M NaCl), 15 mM sodium citrate, pH 7, 2 mM EDTA/0.5% nonfat dry milk (wt/vol), and then hybridized with <sup>32</sup>P-labeled probes (initially probes 2 and 3) under identical conditions for 16 h. Filters were washed for 2 h with several changes of 0.15 M NaCl, 15 mM sodium citrate, pH 7, 0.5% SDS at 68°C and then autoradiographed overnight. Positive pVDRG cosmid clones were isolated through three to four rounds of additional screening.

Human genomic DNA was prepared as described (71). The latter as well as cosmid pVDRG DNA were transferred to nylon membranes, prehybridized for 6 h at 68°C in 6× SSC, and then hybridized with the appropriate <sup>32</sup>P-labeled hVDR cDNA probes overnight. Filters were washed for 2 h as above, and autoradiographed for 4 h (cloned DNA) or 48 h (human genomic DNA). pVDRG DNA was isolated via alkaline lysis and polyethylene glycol precipitation techniques as previously described (71). The isolated DNA was mapped with restriction endonucleases by routine techniques. The relative orientation of specific DNA fragments of related size within the clones was determined by sequential probing with hVDR cDNA probes 1, 2, and 3. DNA fragments that hybridized with the hVDR cDNA probes were isolated, subcloned into the pGEM 3 cloning vector (Promega Biotech, Madison, WI), and then subjected to standard sequencing methods using Sequenase (US Biochemical Corp., Cleveland, OH). The sequencing strategy we employed involved determining the ends of each DNA fragment utilizing the T7 and SP6 sequencing primers followed by extension of this sequence with synthetic oligonucleotides complementary to the newly identified sequence. As the relative position of hVDR gene exons emerged within the DNA clones, synthetic oligonucle-

otides corresponding to hVDR cDNA sequence either 5' or 3' to the identified exon were used as sequencing primers. Intron/exon boundaries were identified using pairs of primers that generated sequence from both strands. Orientation of the exon(s) within the DNA fragment was achieved by identifying sequence overlaps within the clone or by mapping a unique restriction site identified within the newly obtained sequence.

#### hVDR Gene Promoter Analysis

hVDR promoter constructs were prepared beginning with an approximately 3.2 kb SacI-HindIII fragment of the hVDR gene extending from exon 1 upstream approximately -3200 bp. This fragment (pVDE1-3.2) as well as additional fragments were cloned into the HindIII site of the luciferase expression vector pGL-2 basic (Promega). Plasmids pVDE1-2.6, pVDE1-1.93, pVDE1-1.48, pVDE1-1.22, pVDE1-0.59, pVDE1-0.46, pVDE1-0.10, and pVDE1-0.03 were similarly constructed from promoter fragments that contained common 3'-ends but extended 2.6, 1.93, 1.22, 0.596, 0.462, 0.10, or 0.03 kb upstream, respectively. DNA restriction fragments were also isolated from hVDR gene DNA surrounding exon 1C. These fragments include exon 1C and the length of intron sequence lying both upstream and/or downstream of exon 1C as indicated in the figure. The activity of these fragments of DNA was assessed in promoterless pBL-CAT3 and thymidine kinase promoter-containing pBL-CAT2 chloramphenicol acetyltransferase expression vectors (72). The orientation and cloning boundaries of all constructs were verified through DNA sequence analysis.

#### Transient Transfection Analysis of hVDR Promoter Sequences

Hela cells were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and nonessential amino acids. Rat osteosarcoma ROS 17/2.8 cells were grown in Ham's F12 medium supplemented with 10% FCS containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), and nonessential amino acids as previously described. Cells were transfected with DNA 24 h after passage using either polybrene (73) (Sigma Chemical Co., St. Louis, MO) (ROS 17/2.8) or lipofectAMINE (Life Technologies Inc.) (Hela cells). hVDR gene reporter plasmids (5 or 10 µg) were cotransfected together with 1 µg β-galactosidase normalization vector (1 µg), and the activities of the enzymes CAT, luciferase, and/or β-galactosidase were evaluated in cellular extracts prepared 64 h following transfection. Luciferase or CAT activities were determined as previously described (65, 74) and normalized to the activity of β-galactosidase (75). All plasmids used for cellular transfections were purified on QIAGEN ion exchange columns.

#### Acknowledgments

The authors wish to thank Savio Woo and Anthony Dilela for providing the human cosmid library and to acknowledge Clark Huckaby, Bert O'Malley, Shigeaki Kato, and Keiichi Ozono for helpful discussions.

Received February 12, 1997. Revision received March 24, 1997. Accepted March 26, 1997.

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This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education and Science, the Seturo Fujii Memorial, the Osaka Foundation for Promotion of Fundamental Research, Uehara Memorial Foundation, Salt Science Research Foundation, and the NIH.

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## REFERENCES

- Pike JW 1991 Vitamin D<sub>3</sub> receptors: structure and function in transcription. *Annu Rev Nutr* 11:189-216
- Haussler MR, Jurutka PW, Hsieh J-C, Thompson PD, Setnick SH, Haussler CA, Whitfield GK 1995 New understanding of the molecular mechanism of receptor-mediated genomic actions of the vitamin D hormone. *Bone* 17:33S-38S
- Christakos S, Raval-Pandya M, Werny RP, Yang W 1996 Genomic mechanisms involved in the pleiotropic actions of 1,25-dihydroxyvitamin D<sub>3</sub>. *Biochem J* 316:361-371
- O'Malley BW 1990 The steroid receptor superfamily: more excitement predicted for the future. *Mol Endocrinol* 4:363-344
- Beato M, Herrlich P, Schutz G 1995 Steroid hormone receptors: many actors in search of a plot. *Cell* 83:851-857
- Mangelsdorf DJ, Evans RM 1995 The RXR heterodimer and orphan receptors. *Cell* 83:841-850
- Stein GS, Lian JB, Stein JL, Van Wijnen AJ, Montecino M 1996 Transcriptional control of osteoblast growth and differentiation. *Physiol Rev* 76:593-629
- Kerner SA, Scott RA, Pike JW 1989 Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D<sub>3</sub>. *Proc Natl Acad Sci USA* 86:4455-4459
- Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamaki T, Yoshiki S, and Suda T 1981 Differentiation of mouse myeloid leukemia cells induced by 1,25-dihydroxyvitamin D<sub>3</sub>. *Proc Natl Acad Sci USA* 78:4990-4994
- Liu M, Lee M-H, Cohen M, Freedman LP 1996 Transcriptional activation of the p21 gene by vitamin D<sub>3</sub> leads to the differentiation of the myelomonocytic cell line U937. *Genes Dev* 10:142-153
- Huckaby CS, Conneely OM, Beattie WG, Dobson AD, Tsai MJ, O'Malley BW 1987 Structure of the chromosomal chicken progesterone receptor gene. *Proc Natl Acad Sci USA* 84:8380-8388
- Ponglikitmongkol M, Green S, Chambon P 1988 The cloned human estrogen receptor contains a mutation which alters its hormone binding properties. *EMBO J* 7:3385-3388
- Encio IJ, Detera-Wadleigh SD 1991 The genomic structure of the human glucocorticoid receptor. *J Biol Chem* 266:7182-7188
- Marocelli M, Tilley WD, Wilson CM, Griffin JE, Wilson JD, McPhaul MJ 1990 Definition of the human androgen receptor gene structure permits the identification of mutations that cause androgen resistance: premature termination of the receptor protein at amino acid residue 588 causes complete androgen resistance. *Mol Endocrinol* 4:1105-1116
- Lehman JM, Hoffman B, Pfahl M 1991 Genomic organization of the retinoic acid receptor gamma gene. *Nucleic Acids Res* 19:573-778
- Ritchie HT, Wang LH, Tsai S, O'Malley JM 1990 COUP-TF gene: a structure unique for steroid/thyroid receptor superfamily. *Nucleic Acids Res* 18:6857-6862
- Strahle U, Schmidt A, Kelsy G, Steward AF, Cole TJ, Schmid W, Schultz G 1992 At least three promoters direct expression of the mouse glucocorticoid receptor gene. *Proc Natl Acad Sci USA* 89:6731-6735
- Zahraoui A, Cuny G 1987 Nucleotide sequence of the chicken proto-oncogene *c-rbA* corresponding to domain 1 of *v-rbA*. *Eur J Biochem* 165:63-69
- Misrahi M, Looftfelt H, Alger M, Meriel C, Zerah Y, Dessen P, Milgrom E 1988 Organization of the entire rabbit progesterone receptor mRNA and of the promoter and 5' flanking region of the gene. *Nucleic Acids Res* 16:5459-5472
- Lazar MA 1993 Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocr Rev* 14:184-193
- Kastner P, Mark M, Chambon P 1996 Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life. *Cell* 83:859-869
- Beato M, Herrlich P, Schutz G 1996 Steroid hormone receptors: many actors in search of a plot. *Cell* 86:851-857
- Umesono K, Giguere V, Glass CK, Rosenfeld MG, Evans RM 1988 Retinoic acid and thyroid hormone induce gene expression through a common responsive element. *Nature* 336:262-265
- de Thé H, Del Mar Vivanco-Ruiz M, Tiollais P, Stunnenberg H, DeJean A 1990 Identification of a retinoic acid responsive element in the retinoic acid receptor  $\beta$  gene. *Nature* 343:177-180
- Davis KD, Berodini TJ, Stelmach JE, Winkler JD, Lazar MA 1994 Endogenous retinoid X receptors can function as hormone receptors in pituitary cells. *Mol Cell Biol* 14:7105-7110
- Allegretto EA, Sharde N, Zou A, Howell SR, Boehm MF, Hollis BW, Pike JW 1995 Retinoid X receptor acts as a hormone receptor *in vivo* to induce a key metabolic enzyme for 1,25-dihydroxyvitamin D<sub>3</sub>. *J Biol Chem* 270:23906-23909
- Krishnan AV, Feldman D 1991 Cyclic adenosine 3',5'-monophosphate upregulates 1,25-dihydroxyvitamin D receptor gene expression and enhances hormone action. *Mol Endocrinol* 6:198-206
- Krishnan AV, Feldman D 1991 Activation of protein kinase-C inhibits vitamin D receptor gene expression. *Mol Endocrinol* 5:605-612
- Krishnan AV, Cramer SD, Bringham RF, Feldman D 1995 Regulation of 1,25-dihydroxyvitamin D<sub>3</sub> receptors by parathyroid hormone in osteoblastic cells: role of second messenger pathways. *Endocrinology* 136: 705-712
- Levy J, Zull I, Yankowitz N, Shany S 1984 Induction of cytosolic receptors for 1,25-dihydroxyvitamin D<sub>3</sub> in the immature rat uterus by oestradiol. *J Endocrinol* 100:265-269
- Mahonen A, Piskanen A, Maenpaa PH 1991 Homologous and heterologous regulation of 1,25-dihydroxyvitamin D<sub>3</sub> receptor mRNA levels in human osteosarcoma cells. *Biochim Biophys Acta* 108:111-118
- Chen TL, Cone CM, Morey-Holton E, Feldman D 1983 1,25-Dihydroxyvitamin D<sub>3</sub> receptors in cultured rat osteoblast-like cells. Glucocorticoid treatment increases receptor content. *J Biol Chem* 258: 4350-4355
- Petkovich PM, Heersche JNM, Tinker DO, Jones G 1984 Retinoic acid stimulates 1,25-dihydroxyvitamin D<sub>3</sub> binding in rat osteosarcoma cells. *J Biol Chem* 259: 8274-8280
- Chen TL, Feldman D 1985 Retinoic acid modulation of 1,25(OH)<sub>2</sub>D<sub>3</sub> receptors and bioresponse in bone cells. *Biochim Biophys Res Commun* 132:74-80
- Krishnan AV, Feldman D 1991 Stimulation of 1,25-dihydroxyvitamin D<sub>3</sub> receptor gene expression in cultured cells by serum and growth factors. *J Bone Miner Res*

- 6:1099-1107
36. Zhao X, Feldman D 1993 Regulation of vitamin D receptor abundance and responsiveness during differentiation of HT-29 human colon cancer cells. *Endocrinology* 132:1808-1814
37. Costa EM, Hirst MA, Feldman D 1985 Regulation of 1,25-dihydroxyvitamin D<sub>3</sub> receptors by vitamin D analogs in cultured mammalian cells. *Endocrinology* 117:2203-2210
38. Pan LC, Price PA 1987 Ligand-dependent regulation of the 1,25-dihydroxyvitamin D<sub>3</sub> receptor in rat osteosarcoma cells. *J Biol Chem* 262:4670-4675
39. McDonnell DP, Mangelsdorf DJ, Pike JW, Haussler MR, O'Malley BW 1987 Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. *Science* 235:1214-1217
40. Abe J, Morikawa M, Miyamoto K, Kaiho S, Fukushima M, Miyaura C, Abe E, Suda T, Nishii Y 1987 Synthetic analogues of vitamin D<sub>3</sub> with an oxygen atom in the side chain skeleton. A trial of the development of vitamin D compounds which exhibit potent differentiation-inducing activity without inducing hypercalcemia. *FEBS Lett* 226:58-62
41. Binderup L, Latini S, Binderup E, Bretting C, Calverly MJ, Hlansen K 1991 20-Epi-vitamin D<sub>3</sub> analogues: a novel class of potent regulators of cell growth and differentiation. *Biochem Pharmacol* 42:1569-1575
42. Baker AR, McDonnell DP, Hughes MR, Crisp TM, Mangelsdorf DJ, Haussler MR, Shine J, Pike JW, O'Malley BW 1988 Molecular cloning and expression of human vitamin D receptor complementary DNA: structural homology with thyroid hormone receptor. *Proc Natl Acad Sci USA* 85:3294-3298
43. Hughes MR, Malloy PJ, Kieback DG, Kesterson RA, Pike JW, Feldman D, O'Malley BW 1988 Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets. *Science* 242:1702-1705
44. Dielels AG, Woo SL 1987 Cloning large segments of genomic DNA using cosmid vectors. *Methods Enzymol* 152:199-212
45. McDonnell DP, Scott RA, Kerner RA, O'Malley BW, Pike JW 1989 Functional domains of the human vitamin D receptor regulate osteocalcin gene expression. *Mol Endocrinol* 3:635-644
46. Saijo T, Ito M, Takeda E, Huq AHM, Naito E, Yokoto I, Sone T, Pike JW, Kuroda Y 1991 A mutation in vitamin D receptor gene in three Japanese patients with vitamin D-dependent rickets type II: utility of single-strand conformation polymorphism analysis for heterozygous carrier detection. *Am J Hum Genet* 49:668-673
47. Dym WS, Tjian R 1983 The promoter specific transcription factor SP-1 binds to upstream sequences in the SV-40 early promoter. *Cell* 35:79-87
48. Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf JJ, Jonat C, Herrlich P, Karin M 1987 Phorbol ester inducible genes contain a common cis element recognized by a TPA-modulated transacting factor. *Cell* 49:729-739
49. Umesono K, Murakami KK, Thompson C, Evans RM 1991 Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D<sub>3</sub> receptors. *Cell* 65:1255-1266
50. Berg JM 1988 Proposed structure for the zinc binding domains from transcription factor IIA and related proteins. *Proc Natl Acad Sci USA* 85:99-102
51. Mader S, Kumar V, deVerenue H, Chambon P 1989 Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid responsive receptor. *Nature* 338:271-274
52. Hollenberg SM, Evans RM 1988 Multiple and cooperative transactivation domains of the human glucocorticoid receptor. *Cell* 55:899-906
53. Tora L, White J, Brou C, Chambon P 1989 The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* 59:477-487
54. Tora L, Gronemeyer H, Turcotte B, Baub M-P, Chambon P 1988 The N-terminal region of the chicken progesterone receptor specifies target gene activation. *Nature* 333:185-188
55. van Leeuwen JPT M, Birkenhager JC, Vink-van Wijngaarden T, van der Bemd GJC M, Pols HAP 1992 Regulation of 1,25-dihydroxyvitamin D receptor gene expression by parathyroid hormone and cAMP agonists. *Biochem Biophys Res Commun* 185:881-886
56. Van Leeuwen JPT M, Birkenhager JC, Buurman CJ, van der Bemd GJC M, Pols HAP 1992 Bidirectional regulation of the 1,25-dihydroxyvitamin D receptor by phorbol ester activated protein kinase C in osteoblast-like cells: interaction with adenosine 3',5'-monophosphate induced upregulation of the 1,25-dihydroxyvitamin D receptor. *Endocrinology* 130:2259-2266
57. Kadonaga JT, Tjian R 1986 Affinity purification of sequence specific DNA binding proteins. *Proc Natl Acad Sci USA* 83:5889-5893
58. Orkin SH 1995 Transcription factors and hematopoiesis. *J Biol Chem* 270:4955-4958
59. Candelieri GA, Jurutka PW, Haussler MR, St. Arnaud R 1996 A composite element binding the vitamin D receptor, retinoic X receptor  $\alpha$  and a member of the C/EBP-1 family of transcription factors mediates the vitamin D responsiveness of the c-fos promoter. *Mol Cell Biol* 16:584-592
60. Gross C, Eccleshall TR, Mallow PJ, Villa ML, Marcus R, Feldman D 1996 The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. *J Bone Miner Res* 11:1850-1855
61. Arai H, Miyamoto K, Taketani Y, Yamamoto H, Iemori Y, Morita K, Tonal T, Nishishio T, Mori S, Takeda E 1997 A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. *J Bone Miner Res*, in press
62. Kamei Y, Kawada T, Fukuwatari T, Ono T, Kato S, Sugimoto E 1995 Cloning and sequence of the gene encoding the mouse vitamin D receptor. *Gene* 152:281-282
63. Burmester JK, Maeda N, DeLuca HF 1988 Isolation and expression of rat 1,25-dihydroxyvitamin D receptor cDNA. *Proc Natl Acad Sci USA* 85:1005-1009
64. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA 1994 Prediction of bone density from vitamin D receptor alleles. *Nature* 367:284-287
65. Ozono K, Liao J, Kerner SA, Scott RA, Pike JW 1990 The vitamin D responsive element in the human osteocalcin gene. *J Biol Chem* 265:21881-21888
66. Taylor JA, Hirvonen A, Watson M, Pittman G, Mohler JL, Bell DA 1996 Association of prostate cancer with vitamin D receptor gene polymorphism. *Cancer Res* 56:4108-4110
67. Cooper GS, Umbach DM 1996 Are vitamin D receptor polymorphisms associated with bone mineral density? A meta-analysis. *J Bone Miner Res* 11:1641-1649
68. Apte A, Siebert PD 1993 Anchor-ligated cDNA libraries: a technique for generating a cDNA library for the immediate cloning of the 5' ends of mRNAs. *BioTechniques* 15:890-893
69. Chomczynski P, Sacchi N 1987 Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159
70. Gilman M 1987 Preparation and analysis of RNA. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman

- JG, Smith JA, Struhl K (eds) Current Protocols in Molecular Biology. John Wiley & Sons, New York, pp 4.7.1–4.7.8
71. Maniatis T, Fritsch E, Sambrook J 1982 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
72. Luckow B, Schutz G 1987 CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res* 15:5490
73. Kawai S, Nishizawa M 1984 New procedures for DNA transfection with polycation and dimethyl sulfoxide. *Mol Cell Biol* 4:1172–1174
74. Nordeen SK 1988 Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques* 6:454–457
75. Tzukerman MT, Esty A, Santiso-Mere D, Daniellian P, Parker MG, Stein RB, Pike JW, McDonnell DP 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol* 8:21–30
76. Krust A, Green S, Argos P, Kumar V, Walter P, Bomert JM, Chambon P 1986 The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *EMBO J* 5:891–897

